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Up-regulation of Casein-like Proteins during Rat Pancreatic Carcinoma Progression

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ABSTRACT

Through genetic and epigenetic instabilities and phenotypic drift tumour cells alter their expression of surface molecules. Amongst other factors, these changes can give tumour cells the ability to escape from the primary tumour and invade and proliferate at other tissue sites to form metastases. Metastasis formation is directly connected with a poor clinical outcome for cancer patients. By characterising metastasis-associated cell surface molecules and understanding how they facilitate tumour progression one could perhaps later develop therapeutic approaches to inhibit their action. In this thesis I have used subtractive immunisation to immunologically select differences in expression of surface molecules between rat non-metastatic and metastatic tumours. Using cell lines derived from the BSp73 rat pancreatic tumour system four monoclonal antibodies were obtained by subtractive immunisation. The antibodies recognise a group of proteins termed the 10-1 antigen which are dramatically up-regulated on the metastasising 10AS cell line. I have characterised the properties of the 10-1 antigen and amongst other things demonstrate that it is actively secreted from the 10AS cell line in response to changes in growth conditions. This secretion partly explains the observation that the 10-1 antibodies did not inhibit tumour growth and metastasis in spontaneous metastasis assays. The 10-1 antigen is also expressed on a number of other rat tumour cell lines. Purification and microsequencing of the N-terminus of the 10-1 antigen, revealed an amino acid sequence that shares its identity with α-casein proteins found in goat, sheep and bovine species. These data suggest that the protein(s) bearing the 10-1 antigen could be a new rat α-casein protein or casein-like protein. Altered expression of these proteins in rat pancreatic tumour progression and their presence in other neoplasims may be connected to the development of enhanced tumour cell invasion.

Hochregulierung von Casein ähnlichen Proteinen während der Progression des Pankreas Karzinoms in der Ratte

Zusammenfassung

Tumorzellen verändern aufgrund genetischer und epigenetischer Instabilitäten und daraus resultierenden phänotypischen Abweichungen die Expression ihrer Oberflächenmoleküle. Neben anderen Faktoren können diese Veränderungen die Tumorzellen dazu befähigen, aus dem primären Tumor auszuwandern und in andere Gewebe einzudringen, um dort zu proliferieren und Metastasen zu bilden. Die Bildung von Metastasen korreliert direkt mit einer schlechten klinischen Prognose für den Krebspatienten. Durch die Charakterisierung Metastasen-assoziierter Zelloberflächenproteine und durch das Verständnis ihrer Funktion während der Tumorprogression, könnte man eventuell diese Oberflächenproteine als Ziel für einen therapeutischen Ansatz verwenden. In dieser Doktorarbeit habe ich die Methode der subtraktiven Immunisierung benutzt, um die Unterschiede in der Expression von Oberflächenmolekülen zwischen nicht-metastasierenden und metastasierenden Rattentumoren mittels subtraktiver Immunisierung zu ermitteln. Subtraktive Immunisierung zwischen zwei Zelllinien, die aus dem BSp73 Ratten-Bauchspeicheldrüsen-Tumormodell stammen, ergab vier monoklonale Antikörper. Diese Antikörper erkennen eine Gruppe von Proteinen, die 10-1-Antigene, welche in der metastasierenden 10AS-Zelllinie dramatisch hochreguliert sind. Ich habe die Eigenschaften des 10-1-Antigens charakterisiert und konnte zeigen, dass es von der 10AS-Zelllinie aktiv als Antwort auf veränderte Wachstumsbedingungen sezerniert wird. Diese Sekretion des Antigens ist eine möglich Erklärung für die Beobachtung, dass die 10-1-Antikörper weder das Tumorwachstum noch die spontane Metastasierung in entsprechenden Metastasierungsassays inhibieren. Aufreinigung und Mikrosequenzierung des N-terminalen Endes des 10-1-Antigens ergaben eine Aminosäuresequenz, die mit den bekannten α-Casein-Proteinen aus Ziege, Schaf und Rind übereinstimmt. Diese Daten legen nahe, dass die Proteine, die das 10-1 Antigen tragen, das entprechende homologe Protein in der Ratte oder ein neues Casein-ähnliches Protein sein könnten. Veränderte Expression dieser Proteine während der Progression von Ratten-Bauchspeicheldrüsen-Tumoren und ihre Anwesenheit auch in anderen Tumormodellen könnten im Zusammenhang mit der Entwicklung verstärkter Tumorzellinvasion stehen.

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Abbreviations

A	Adenosine
APS	Ammonium persulfate
AMF	Autocrine motility factor
bp	Base pair
BSA	Bovine serum albumin
C	Cytidine
^{o}C	Degrees celsius
C-CAM	Epithelium specific adhesion molecule
CEA	Carcinoembryonic antigen
CD	Cluster of differentiation
CD44s	CD44 standard protein
CD44v	CD44 variant protein
cDNA	copy DNA
cm	Centimeter (10^{-2} meter)
DMEM	Dulbecco's modified eagles medium
DMF	N,N'-Dimethylformamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
ECM	Extracellular martix
EDTA	Ethylendiamine-N,N-tetracetate
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
et al	and others (Lat. *et alii*)
ELISA	Enzyme-linked-immunoabsorbent-assay
FACs	Fluorescence activated cell sorter
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FPLC	Flow pressue liquid chromatography
g	Gram
G	Guanosine
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	Granulocyte/macrophage colony stimulating factor
GSL	Glycosphingolipids
GPI	Glycosylphosphatidylinositol
HA	Hyaluronic acid
HGF/SF	Hepatocyte growth factor/scattor factor
hr	Hour
HRP	Horseradish peroxidase
ICAM	Intercellular adhesion molecule
Ig	Immunoglobulin

IGF	Insulin-like growth factor
IgSF	Immunoglobulin superfamily
IL	Interleukin
INF	Interferon
i.p	Intraperitoneal
kb	Kilobase (1kb=1000bp)
L	litre
Le^x	Sialyl $Lewis^x$
Le^a	Sialyl $Lewis^a$
kDa	Kilodalton (10^3 daltons)
m	milli
M	Molar
mA	Milliampere
Mab	Monoclonal antibody
mg	Milligram (10^{-3} gram)
min	Minute
ml	Millilitre (10^{-3} l)
MMP	Matrix metalloproteinases
mRNA	Messenger RNA
MSF	Migration stimulating factor
μ	Micro-
μg	Microgram 10^{-6} gram)
μl	Microliter (10^{-6} l)
mM	Millimolar (10^{-3} molar)
μM	Micromolar (10^{-6} molar)
n	Nano-
N-CAM	Neural crest adhesion molecule
ng	Nanogram (10^{-9} gram)
nm	Nano meter (10^{-9} meter)
OD	Optical Density
p	Pico-
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-ECGF	Platelet derived endothelial cell growth factor
PDGF	Platelet derived growth factor
PeCAM	Platelet endothelial adhesion molecule
PEG	Polyethylene glycol
pg	Picograme (10^{-12} gram)
RGD	Arg-Gly-Asp
RNA	Ribonucleic acid
rpm	Revolutions per minute
RTKs	Receptor tyrosine kinases
RT-PCR	Reverse transcription PCR

sec	Second
SDS	Sodium-Lauryl-Sulfate (sodium dodecyl sulfate)
Stds	Standards
T	Thymidine
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF-α	Transforming growth factor alpha
TGF-β	Transforming growth factor beta
TNF-α	Tumour necrosis factor-alpha
tPA	Tissue-type plasminogen activator
TRIS	Tris-(hydroxymethyl)-aminomethane
U	Unit(s)
uPA	Urokinase plasminogen activator
UV	Ultraviolet
v	Variant
VEGF/VPF	Vascular endothelial factor/vascular permeability factor
V	Volt
VCAM	Vascular adhesion molecule
W	Watt

Amino acids

A	Ala	Alanine
C	Cys	Cystine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	lleu	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

INTRODUCTION

At the present moment in the western world a large proportion of clinical research is concerned with gaining a better understanding of cancer. Cancer is responsible for many early mortalities in our society, and as such has profound effects on the productive capacity of our communities. Thus, there are many vested interests ranging from governments, health policy makers and pharmaceutical companies who are seeking a cure for this disease. It is the formation of metastasis, by which malignant tumour cells leave their primary site and spread to distant locations throughout the body, that makes cancer such a lethal disease.

I have utilised the experimental technique "subtractive immunisation" to identify and characterise new surface antigens that may have a role in metastasis. In this way monoclonal antibodies were produced that selectively bind to antigens expressed on metastatic cells of a rat pancreatic carcinoma model. These antigens are secreted or shed from the pancreatic carcinoma cells and are also present on other tumour cell lines. As an introduction to this thesis, I would therefore like to introduce the current literature about molecules that have been implicated to play a functional role in metastasis.

Cancer and metastasis defined

Cancer may be succinctly defined as the cellular loss of proliferative and spatial control (Schwarz et al., 1988). This definition specifies that tumour cells differ from non-transformed cells in their proliferative capacity, as well as their ability to invade adjacent tissue compartments and metastasise. In order for a tumour to become metastatic, it is has to acquire a number of properties that allow it to detach from the primary tumour, migrate through the basement membrane and extracellular matrix, extravasate and travel in the circulatory system to a new site, intravasate through a vessel wall, and through

proliferation and the induction of angiogenesis establish growth at a secondary site. The process of adhesion, proteolysis and migration constitutes invasion and has become to be known as the "metastatic cascade" (Table 1; Hart et al., 1989). Failure of the tumour to initiate any of these events thwarts metastasis formation.

Table 1. The metastatic cascade.

Initial transforming event
↓
Growth of neoplastic cells
↓
Neovascularisation/angiogenesis of the tumour
↓
Detachment of neoplastic cells from primary tumour
↓
Local invasion of extracellular matrix by tumour cells
↓
Intravasation of tumour cells into lymphatics or vasculature
↓
Survival of tumour cells in circulation and
avoidance of immunological attack
↓
Extravasation of tumour cells from vasculature
into secondary organ tissue
↓
Survival and proliferation with organ parenchyma

Taken from Ahmad and Hart, (1996).

Invasion

For tumour cells to invade, the three steps of proteolysis, changes in adhesive properties and migration must be fulfilled. Proteolysis is needed to traverse ECM barriers in the interstitial stroma and basement membrane. Tumour cells perform this by producing and secreting proteases themselves, by stimulating surrounding cells to produce proteases, or by producing factors to activate existing local proteases. Changes in adhesive properties occur between tumour cells themselves, and between tumour cells and stromal cells, endothelial cells, and the interstitial stroma and basement membrane. These interactions are mediated by cell adhesion molecules in the tumour or metastatic site, and in the vasculature. Migration represents the forward motion of cells in the form of a pseudopodial protrusion followed by translocation and retraction of the trailing cell body (Price et al., 1997). Cancer cell migration is stimulated through paracrine and autocrine motility factors. It is through the net balance of these three actions, proteolysis, changes in adhesive properties and migration in the cellular environment that the invasive process, either physiological or malignant, is driven.

Cell adhesion molecules

Adhesion molecules are intrinsically involved in the processes of metastasis. Metastasis cannot occur if the tumour cells are tightly bound by their adhesion molecules to other cells in the primary tumour (Van Roy et al., 1992). Therefore in the early stages of metastasis there is an important change in the phenotype of some tumour cells from being stationary and tightly adhered, to losing adhesion properties (Jouanneau et al. 1991) and thereby gaining the potential to migrate to and bind the basement membrane of circulatory vessels. Once in the circulatory system, in order to form metastatic lesions the tumour cells must again possess different adhesive properties. These allow them to bind to and traverse the basement membrane of the

capillary endothelium, where they lodge and migrate further into the tissue region. These processes all involve adhesion molecules that have specific roles in the metastatic process. For example, through homotypic interactions cadherins stabilise normal tissue integrity, whereas loss of these cell surface molecules has been associated with increased metastatic potential (Behrens, 1993). Heterotypic interactions occur between tumour cells and endothelial cells as they enter and exit the vasculature. Migration and organisation of tumour cells may be regulated by interactions with ECM proteins through cell adhesion molecules such as the integrins (Hynes, 1992). The cell adhesion molecules involved in these interactions can be divided into the molecular families of cadherins, integrins, the immunoglobulin superfamily, laminin binding proteins, mucins and CD44 (Table 2). A short summary of the major characteristics of these molecules will now be given.

Cadherins

Cadherins are a family of cell surface molecules that mediate homophilic interactions between cells and are chiefly responsible for establishing and maintaining intercellular interactions (Takeichia, 1988). Structurally, the cadherins are composed of several distinct domains: an extracellular region composed of 4 repeat domains 110 amino acids in length that are important for calcium binding and cell-cell adhesion (Nose et al., 1990), a small transmembrane domain and a highly conserved cytoplasmic domain that is associated with accessory cytoplasmic proteins (α,β,γ-catenins, Ozawa et al., 1989) needed for complete cell-cell adhesion (Takeichi, 1991). There are at least three subclasses of cadherin, E (epithelial)-cadherin, P (placental)-cadherin, and N (neural and muscular)-cadherin. E-cadherin is expressed in almost all epithelia and plays a pivotal role in the maintenance of epithelial structure, and as such has been studied extensively with regard to tumorigenesis and metastasis (Takeichia, 1991; Gumbiner et al., 1988; Grunwald, 1993).

Table 2. Cell adhesion molecules involved in metastasis.

Family	Receptor	Ligand	Distribution on normal tissue
Integrins	Table 3		
IgG superfamily	ICAM-1	$\alpha_L\beta_2$	Leukocytes
	ICAM-2	$\alpha_L\beta_2$	Endothelial cells
	ICAM-3	$\alpha_L\beta_2$	Leukocytes
	PeCAM-1		Most immune cells, platelets, monocytes, neutrophils, all vascular endothelial cells
	LFA-2	LFA-3	T-cells
	LFA-3	LFA-2	Widespread
	VCAM-1	$\alpha_4\beta_1$	Activated endothelial cells
	Ca^{2+} independent CAMs	Homophilic binding	Brain, muscle, heart, kidney
	N-CAM	Neural and gial cells	?
	Ng-CAM	Homophilic and heterophilic binding	?
Cadherin	N-cadherin	Homophilic binding	Neural cells, brain, muscle, lens
	E-cadherin	Homophilic binding	Epithelium
	P-cadherin	Homophilic binding	Placenta, epithelium, mesothelium
Selectin	L-selectin	Carbohydrate	Lymphocytes
	E-selectin	Sialyl-Lewis X	Endothelial cells, neutrophils, tumour cells
	P-selectin	Lewis X	Platelets, neutrophils, monocytes, endothelial cells
CD44	CD44 and variant isoforms	Hyaluronic acid, laminin, collagens, fibronectin	Widespread
Laminin binding proteins	67LR	Laminin	Basement membranes

Adapted from Streit et al. (1996).

The detachment of cancer cells from the primary tumour is the initial step in the tumour cascade and this is caused by the disruption of cell-cell connections (Van Roy et al., 1992). Consequently, a reduction in cadherin expression is necessary and can occur either by down regulation of expression or by loss of protein function. Various authors have shown that a decrease or absence of E-cadherin expression in epithelial tumour cells leads to an increased invasive potential and is associated with the progression of a variety of epithelial neoplasias (Frixen et al., 1991; Matsuura et al., 1992; Giroldi et al., 1994; Shiozaki et al., 1996). Transfection of E-cadherin into highly invasive epithelial tumour cells reduced their invasiveness, and treatment of these transfectants with anti-E-cadherin antibodies reintroduced the invasive phenotype (Vleminckx et al., 1991). In the same study, E-cadherin-specific antisense RNA was transfected into noninvasive *ras*-transformed epithelial cells, which made these cells invasive. A recent report by Perl et al. (1998) demonstrated *in vivo* that the loss of E-cadherin expression is a rate-limiting step in the progression of adenoma to carcinoma. These studies suggest that impairment of E-cadherin function could contribute to the escape of cancer cells from the primary tumour, and in as much be a trigger for invasion and metastasis (Shiozaki et al., 1996).

In recent years, various studies have been performed to study E-cadherin expression in many different human cell lines. These studies found that cell lines with an epithelioid morphology were generally noninvasive and expressed E-cadherin, whereas cell lines with a fibroblastoid morphology were invasive and had often lost E-cadherin expression (Frixen et al., 1991). A number of investigators have observed a correlation between reduced E-cadherin expression and poor differentiation of human tumours (Birchmeier et al., 1994), thus indicating a role for E-cadherin in the maintenance of a differentiated phenotype in carcinomas. A correlation between reduced E-cadherin expression and poor clinical outcome has been observed in prostate and bladder cancer (Birchmeier et al., 1994; Umbas et al., 1994), suggesting that E-cadherin may be a marker of prognostic value. In renal-cell cancer,

decreased E-cadherin expression has been associated with the occurrence of metastasis, giving a poor prognosis (Katagiri et al., 1995). These studies further support the notion that E-cadherin is an invasion-suppressor protein.

The literature concerning studies of P- and N-cadherins with metastasis is not so extensive. These cadherins have been identified on melanoma cells, but no mechanism for their involvement in metastasis has been described (Matsuyoshi et al., 1997). In certain invasive breast cancer cell lines upregulation of N-cadherin was observed, which promoted their interaction with stromal cells, suggesting that N-cadherin facilitates invasion and metastasis (Hazan et al., 1997). This is in contrary to observations concerning E-cadherin where downregulation is associated with a metastatic phenotype. Further studies will be required to determine what is the mechanism for the other cadherins involvement in metastasis.

Integrins

Integrins are transmembrane glycoproteins composed of α and β subunits that are nonconvalently associated to form a heterodimeric complex (Hynes, 1992). At least 15 different α-chains and 9 different β-chains have been identified, and these can associate in a variety of combinations to form many different heterodimers (Table 3). They are expressed at a high density on the cell surface and predominantly mediate cell-matrix interactions during cell migration (Albelda et al., 1990a), and to a lesser degree heterophilic cell-cell adhesion (Springer, 1990). The combination of alpha and beta chains dictates to an extent their ligand specificity. Cells expressing the appropriately paired subunits can bind to a variety of extracellular protein ligands, such as fibronectin, laminin, tenascin, thrombospondin, vitronectin, and collagens (Hynes, 1992). Cells that express multiple integrin forms on their surface have the capacity to recognise multiple ECM components, providing positional information needed for anchorage, polarity, differentiation, and directed migration. This recognition system is utilised by leukocytes in extravasation at

Table 3. Association of α and β integrins, and their ligands.

Integrin β-subunit	Integrin α-subunit	Ligands
β_1	α_1	Collagens, laminins
	α_2	Collagens, laminins, fibronectin, echovirus 1
	α_3	Fibronectin, collagen 1, epiligrin, invasin
	α_4	Fibronectin, epiligrin, VCAMs, invasin
	α_5	Fibronectin, RGD sequence, invasin
	α_6	Laminin, invasin
	α_7	Laminin
	α_8	?
	α_9	Tenascin, collagen, laminin
	α_v	Fibronectin, RGD sequence, vitronectin
β_2	α_L	Fibrinogen, C3bi
	α_M	Fibrinogen, factor X, ICAMs
	α_X	Fibrinogen, C3bi
β_3	α_{IIB}	Collagens, vitronectin, fibronectin, RGD sequence, fibrinogen, v. Willebrand factor, disintegrins thrombospondin, Borrelia burgdorferi
	α_v	Fibronectin, RGD sequence, vitronectin, fibrinogen, thrombospondin, osteopontin, cytoactin/tenascin, disintegrins, v. Willebrand factor, HIV Tat proteins
β_4	α_6	Laminin
β_5	α_v	Vitronectin, RGD sequence, HIV Tat proteins
β_6	α_v	Fibronectin, cytoactin/tenascin
β_7	α_4	Fibronectin, mucosal ACAM-1, VCAMs
β_8	α_v	?

Adapted from Ahmad and Hart, 1996; Streit et al. (1996); Gille and Swerlick, (1997).
ACAM Addressin cell adhesion molecule-1; C3bi Complement factor C3.

sites of inflammation and is probably used similarly by tumour cells in cancer cell extravasation (Heino, 1993; Herzberg et al., 1996; Hynes 1992).

It is through their interactions with ECM components that integrins were initially suspected to be involved in promoting tumour growth and metastasis. Numerous studies have revealed that differential integrin expression on tumours is associated with metastatic behaviour (Albelda, 1993b). For example, Danen et al. (1993a; 1993b) showed that upregulation of $\alpha_2\beta_1$, $\alpha_3\beta_1$

and $\alpha_6\beta_1$ in human melanomas correlates with metastatic potential and cell migration. Studies of integrin expression in normal colon, adenomas and carcinomas within the same patient showed that as cells progress from adenomas to carcinomas they loose expression of $\alpha_3\beta_1$, (receptor for collagen and laminin) and $\alpha_5\beta_1$ (receptor for fibronectin, Pignatelli et al., 1990; Stallmach et al., 1992). Benign tumours of the head, neck and skin down-regulate α_6 integrin expression, whereas elevated levels are found on metastatic tumours (Friedrichs et al., 1995a). In addition, patient survival from these tumours correlates with loss of α_6 expression. These observations suggest that through their ECM ligand interactions, integrins can regulate tumour behaviour.

Integrins bind to short peptide sequences on their ligands. Several recognise the tripeptide Arg-Gly-Asp (RGD) that is expressed in fibronectin, collagen, invasin, laminin and vitronectin (Gille and Swerlick, 1997; Varner and Cheresh, 1996). Studies interfering with integrin binding to this peptide sequence have provided strong evidence for the ability of integrins to regulate metastasis. For example, Humphries et al.(1986) used short peptides to block the RGD ligand binding site. In this model, coinoculation of the tumour cells and peptides into nude mice resulted in a reduction of murine melanoma lung metastases. Similarly, RGD peptides have been used to block *in vitro* invasion of basement membrane matrices (Gehlsen et al., 1988). Ruiz et al. (1993) blocked metastasis of B16 murine melanoma cells with antibodies to $\alpha_6\beta_4$. Treatment of metastatic cells with antibodies against α_6 , or suppression of α_6 expression by ribozyme treatment resulted in a reduction of adhesion, proteolysis and experimental metastasis (Blood and Zetter, 1993; Ruiz et al., 1993). These studies demonstrate that interference with integrin binding is potentially a potent therapeutic strategy to combat metastasis.

Brooks et al. (1996) have described an association between integrins and metalloproteinases (MMPs). They demonstrated that the proteolytically active form of the metalloproteinase MMP-2 can bind to integrin $\alpha_v\beta_3$, and can thus be localised on the surface of metastatic tumour cells. This localisation

provides migratory cells with the ability to coordinate matrix degradation and cellular motility, thereby facilitating tumour cell invasion. The integrins $\alpha_2\beta_1$ $\alpha_5\beta_1$ and $\alpha_4\beta_1$ have also been associated with the ligation of MMPs (Varner and Cheresh, 1996), suggesting that other family members may also have diverse roles of facilitating tumour cell adhesion, motility, and ECM proteolysis.

Immunoglobulin superfamily

The immunoglobulin superfamily (IgSF) is composed of a large number of transmembrane-bound calcium-independent molecules that are capable of both heterophilic and homophilic interactions. Structurally they feature an extracellular domain of multiple immunoglobulin-like homology units (Ig) and fibronectin III repeats (FNIII, Springer, 1990). The Ig units are 70-110 amino acids long, organised into several β-pleated sheets and each stabilised by a single disulfide bond. Both the Ig and FNIII motifs have been shown to be involved in cell-cell interactions. The family includes molecules such as major histocompatibility molecules (CD2, CD4, CD8 and the T-cell receptor), and those involved with leukocyte trafficking [intercellular adhesion molecule (ICAM-1), ICAM-2, ICAM-3 and vascular adhesion molecule (VCAM)], as well neural crest adhesion molecule (N-CAM), platelet endothelial adhesion molecule PeCAM-1, epithelium-specific adhesion molecule (C-CAM), and carcinoembryonic antigen (CEA) (Pignatelli et al., 1994; Yeatman et al., 1993; Zetter, 1993). Cytokines can upregulate the expression of certain IgSFs on some cell types, and clearly there is an implication for this relationship in tumour-stroma interactions.

Increased expression of ICAM-1 on human melanoma and lymphoma is associated with increased metastatic spread, a high probability of remission after surgery (Johnson et al., 1989; Natali et al., 1990; Stauder et al., 1989), and poor prognosis for patients with haemopoietic and epithelial tumours (Johnson et al., 1989; Natali et al., 1990; Huang et al., 1995; Santorsa et al., 1995). The interaction of tumour-expressed ICAM-1 with its endothelial

integrin ligand $\alpha_L\beta_2$ (LFA-1) is in some tumour models partly responsible for their metastatic properties. Antibodies to ICAM-1 or $\alpha_L\beta_2$ have been shown to inhibit invasion and metastasis of murine and human lymphomas (Harning et al., 1993; Rocha et al., 1996). Similar antibodies have also suppressed the growth of more advanced tumours (Huang et al., 1995). These observations suggest that the antibodies affect the interaction of ICAM-1 and $\alpha_L\beta_2$, by interfering with the initial attachment of tumour cells to the endothelium, and in more advanced tumours by disrupting ICAM-1´s homophilic connections. These studies indicate that ICAM-1 can have the dual roles of mediating metastatic tumour-cell adhesion to the endothelium and invasion of anatomical sites.

Other IgSFs have similar ligand-dependent metastatic-associations. VCAM-1 is expressed on endothelial cells stimulated by certain cytokines, and has been implicated in metastasis via the binding of its ligand $\alpha_4\beta_1$ integrin (VLA-4) present on tumours that have the propensity for haematogenous metastasis (melanoma, osteosarcoma and thyroid carcinoma; Albelda et al., 1990b). N-CAM, an adhesion molecule expressed on neural crest derived-cells, has been shown to be a sensitive marker for tumour grade and prognosis in small cell lung cancer (Pujol et al., 1993; Michalides et al., 1994). However, the mechanism for N-CAMs involvement in tumorigenesis has not been determined.

The carcinoembryonic antigen (CEA) was discovered in 1965 and refers to a large and complex family of abundantly expressed adhesion molecules (Gold and Freedman, 1965). It is 200 kDa glycoprotein containing about 60% carbohydrate and can either be membrane bound or associated with the plasma membrane via a glycosylphosphatidylinositol (GPI) anchor. Adhesion of CEA molecules is primarily by homophilic binding to molecules on adjacent cells. In the adult CEA-related cell surface molecules are primarily expressed in various epithelia, vessel endothelia, and haematopoietic cells. They are also expressed during development, and are for example observed in placental

trophoblasts, muscle development, and the development and eruption of teeth (reviewed in Öbrink, 1997).

CEA is the most widely used tumour marker. Increased levels have been found in a number of human carcinomas, and it is particularly useful for monitoring patients diagnosed with colorectal cancer (Moertel et al., 1993). In normal colonic cells and differentiated colon carcinomas the distribution of CEA is apical, whereas in undifferentiated colonic tumours it is equally expressed over the entire cell membrane (Jessup and Thomas, 1989). Other studies have shown CEA to mediate homotypic aggregation of cancer cells and mediate homing of colorectal cancer cells to the liver (Johnson, 1991; Jothy et al., 1995), suggesting a direct role for it in tumorigenesis and metastasis. However, despite the enormous amount of literature that has described an association of CEA with malignancy, no clear picture of a functional role for it in metastasis has emerged.

Selectins

Selectins are cell adhesion molecules of the immunoglobulin family that mediate interactions between leukocytes and vessel walls, for example, at sites of tissue injury and inflammation. Three selectin family members have been described with differential expression on leukocytes, (L-selectin), platelets, (P-selectin) and vascular endothelium, (E-selectin; Springer and Lasky, 1991), each of which contain a lectin-like domain capable of binding to specific carbohydrate structures, such as the sialyl Lewis^x (Le^x), sialyl Lewis^a (Le^a) antigens, fucosylated molecules and heavily O-glycosylated proteins known as mucins (Shimizu and Shaw, 1993). Physiologically, selectins encourage vascular adhesion by mediating neutrophil, monocyte and lymphocyte rolling, in recruiting lymphocytes to inflammation sites, in activating neutrophils and in lymphocyte homing (Crokett-Torabi and Fantone, 1995). Their importance in metastasis is due to their association with the binding of metastatic cells to the endothelium via tumour expressed carbohydrate antigens during the entry

and exit of tumour cells from the circulatory system. This will be presented in later sections.

Laminin receptors

Laminin is a high molecular weight glycoprotein and a major component of basement membranes. It has several important biological properties, such as the stimulation of cellular attachment, differentiation, proliferation, migration, and neurite outgrowth (Beck et al., 1990). Furthermore, for tumour cells to enter and exit the circulatory system they need to bind to and penetrate through basement membranes. Not surprisingly, receptors for laminin such as members of the integrin family have been implicated in metastasis. A number of laminin-binding proteins have been isolated, including the high-affinity 67 kDa receptor (67LR), members of the galectin family, and integrins. 67LR is the best described and has been shown to be associated with the invasive and metastatic phenotype of cancer cells (Sobel, 1994 and Castronovo, 1993), and poor prognosis (Martignone et al., 1993). On normal cells, 67LR is present on their basal surface in contact with the basement membrane. In contrast, on tumour cells it is increased in expression, disorganised in distribution, and unoccupied by ligand (Wewer et al., 1986). Observing this, several authors found a correlation between 67LR expression and the metastatic potential of colon, breast, ovarian and gastric cancer (Castronovo, 1993; Daidone et al., 1991; Demeter et al., 1992; D`Errico et al., 1991; Sobel, 1994; Terranova et al., 1983). Cioce et al. (1991) observed a higher level of laminin receptor expression in colon cancer metastases than in the primary tumour lesion, suggesting that increased expression of the 67LR receptor is associated with a more invasive phenotype and a higher metastatic potential. Utilising a similar model, Aznavoorian et al. (1990) observed that subclones of a human colon carcinoma cell line which varied in their invasive and non-invasive properties differed mainly in their ability to interact with basement membrane protein laminin. The invasive subclone expressed higher levels of laminin receptors

and had more enhanced laminin-mediated adhesion, spreading and migration than the noninvasive clone. *In vivo* data supports a role for the laminin receptor in metastasis. Bersalier et al. (1995) inhibited liver colonisation of human colorectal cancer cells using a pentapeptide from the laminin-β_1 chain which blocked laminin binding to the 67LR receptor, whereas a peptide from the laminin-α_1 chain promoted liver metastasis formation by stimulating homotypic cell aggregation into tumour emboli. These studies suggest that tumour cell adhesion and migration is enhanced by the binding of the 67LR receptor to laminin, and constitutes an important mechanism in metastasis formation.

CD44

CD44 is a widely expressed cell surface glycoprotein that is structurally related to several hyluronate binding proteins, and serves as an adhesion molecule in cell-substrate and cell-cell interactions (Underhill, 1992). The molecule has been shown to have a variety of functions, including binding to extracellular matrix hyaluronic acid (HA), fibronectin, laminin, collagen, chrondroitin sulphate-modified invariant chain, osteopontin and serglycin. Physiologically it has been implicated in lymphocyte rolling, homing and aggregation, lympho-haematopoiesis, T cell activation, limb outgrowth, and cell migration and tumour metastasis (Hua-Xin et al., 1998; Price et al., 1997; Sherman et al., 1998; Galluzzo et al., 1995; reviewed recently in Sherman, 1996; Günthert, 1996; Zöller, 1996; Naor, 1997).

The numerous functions and molecular partners of CD44 can be related to its complex structure. The diversity is partly created by the incorporation of amino acid stretches encoded by ten alternatively spliced exons that can be inserted into one single extracellular site located close to the transmembrane domain to create CD44 variants (CD44v, Jackson et al., 1992). To further increase the complexity of CD44v, the primary structure is modified with various glycans, such as N- and O-linked glycosylations, chrondroitin and

heparan sulfate side chains (Günthert, 1996). These modifications can also be cell-type specific (Brown et al., 1991; Hofmann et al., 1993). The most common and widely expressed 85-kDa (CD44s) form does not include any variant exons. It has a virtually ubiquitous expression pattern, while CD44v forms are only expressed in a select number of tissues and tumours (reviewed in Naor, 1997).

CD44 binds to a wide range of ligands including ECM components, chemotactic cytokines such as osteopontin and growth factors. It is not surprising therefore that there is much evidence to suggest that it plays a role in metastasis. I will firstly present those studies that have revealed interesting correlations with CD44 expression and metastatic progression, and thereafter consider the mechanisms by which CD44 could confer metastatic behaviour on tumour cells.

CD44 has been studied in many different tumour types and I will limit my discussion to some of the most important examples. CD44 is involved in leukocyte development and activation (Zöller, 1996), and not surprisingly CD44 expression is implicated in the progression of non-Hodgkin´s lymphomas (NHLs). Immunohistochemical studies revealed a poor prognosis for patients expressing elevated levels of CD44s and *de novo* expression of v3 and v6 variants (Stauder et al., 1995). It has been speculated that NHLs share the properties of activated lymphocytes, specifically that they utilise v3 and v6 variants to promote entry into and/or expansion into the lymphatic tissue (Herrlich et al., 1993).

Dall et al. (1996) revealed by immunostaining that during the progression of uterine cervical carcinoma from low-grade squamous intraepithelial stage to high-grade squamous intraepithelial stage and invasive stage there was an increase in CD44v7/8 expression. This expression pattern also equated with shorter survival (Kainz et al., 1995) and suggests that these variants could play a role in metastasis of cervical carcinoma.

In studies considering colorectal and breast cancer initially conflicting data emerged. For colorectal cancer, Mulder et al. (1994) observed a

correlation of CD44v6 presence with poor patient outcome, whereas Koretz et al. (1995) found no such correlation. Evaluation of CD44v6 in breast revealed similar inconsistencies. Kaufmann et al. (1995) described CD44v6 reactivity as an indicator for adverse prognosis, while Friederichs et al. (1995b; 1995c) found no correlation of v6 expression with survival. These discrepancies are related to technical differences or to patient selection criteria used for the studies. In a recent study by Wielenga et al. (1998) a panel of different antibodies against CD44v6 reconfirmed CD44v6 as a prognostic marker for colorectal cancer, and convincingly demonstrated that the affinities of the antibodies used is critical.

Studies of the central nervous system have also suggested a role for CD44. For example, Sherman et al. (1995, 1997) showed by histochemistry and RT-PCR that human schwannomas express higher total levels of CD44s and additional splice variants, suggesting that CD44 could be a marker for malignant transformation of schwann cells. In neuroblastomas CD44s is down regulated (Shtivelman and Bishop, 1991) and variant isoforms have not been detected at any stage of the disease (Terpe et al., 1995). Furthermore, CD44s expression and the absence of the neuroblastoma maker MYCN proto-oncogene correlates as a favourable prognostic factor (Combaret et al., 1995; Christiansen et al., 1995; Terpe et al., 1995). These findings suggest that the absence, rather than the presence of CD44 is associated with a poor outcome for neuroblastoma patients.

These data suggest that CD44 protein expression can both positively and negatively influence metastatic progression, and that different alternatively spliced exons may play roles in different tumours. Due to CD44s being the principle receptor for HA (Aruffo et al., 1990), most work on CD44 and its function in metastasis has focussed on this interaction. Very little attention has been paid to the other ligands to which CD44 binds.

Thomas et al. (1992) showed that human melanoma cells expressing high levels of CD44s which had a high HA binding capacity had increased motility, homotypic aggregation and an increased invasive potential in vivo compared to

cells expressing low levels of CD44s (Birch et al., 1991). In support of this idea Guo et al. (1994), established two sublines of this human melanoma cell line, which differed in CD44s expression. The cell line generated from the invading lymph node which expressed CD44 and bound HA was able to form metastases. In comparison the other established from the primary tumour which did not express CD44 failed to generate metastases, suggesting but not proving, that CD44-HA interaction influence the invasion of human melanoma metastatic cells at secondary sites.

There is a great deal of experimental evidence for the involvement of CD44 variants in metastasis. The work of Günthert et al. (1991) is perhaps the best confirmation of the malignant potential of CD44. They revealed that artificial overexpression of a splice variant v4-v7 isoform of CD44 promoted the metastatic spreading of a non-metastatic rat pancreatic carcinoma cell line. An antibody against CD44v6 blocked this process, suggesting that this CD44 splice variant binds to some unknown ligand and thereby potentiates the metastatic potential of these cells (Reber et al., 1990; Seiter et al., 1993). Corresponding overexpression of CD44s in the non-metastatic tumour cells did not induce metastasis (Rudy et al., 1993), suggesting that in this model CD44s is not involved in the dissemination process. It was speculated that the metastatic cells exploit a normal physiological function that is dependent on CD44v expression (Arch et al., 1992; Moll et al., 1996).

In the pancreatic carcinoma system utilised by Günthert et al. (1991) overexpression of CD44s did not induce metastasis formation. Sleeman et al. (1996) observed that CD44v4-v7 formed clusters and increased their HA binding capacity when transfected into these cells. They then considered whether CD44v4-v7-HA interactions were responsible for metastasis in this system. Transfectants expressing a hyaluronidase on their surface no longer bound HA, but they still formed tumours and metastasised (Sleeman et al., 1996), confirming that CD44v-HA interactions in this system were not necessary for metastasis formation and that the action of CD44 variants was through some other unknown ligand.

CD44 variants do not seem to promote metastasis in every system. For example, melanoma cells transfected with CD44s had increased tumour growth in immunodeficient mice as compared with cells expressing CD44E (epithelial CD44, expressing variant exons v8-v10, Bartolazzi, et al., 1995). Similarly, Namalwa cells (Burkitt lymphoma cells) transfected with CD44s exhibited strong tumour and metastasis formation, but had a reduced ability to develop tumours when transfected with CD44E or CD44v6-v10 (Sy et al., 1991; Bartolazzi, et al., 1995). It was also observed that melanoma or Namalwa cells expressing CD44 variants, or CD44 mutants that do not bind HA, were unable to grow efficiently in immunodeficient mice, whereas cells expressing CD44s that bound HA developed tumours and metastasis (Sy et al., 1991; Bartolazzi, et al., 1994, 1995; Walter et al., 1995). These studies suggest that CD44 variants in these systems are not responsible for generating metastasis, rather that the tumour-metastasising promoting properties can be attributed to the ability of CD44s to mediate cellular adhesion via HA. In summary, expression of CD44s and CD44v has varied effects on metastatic proclivity in different systems, and their action through ligand binding or associations with other molecules has not yet been fully elucidated.

Proteolysis

During the process of cancer invasion and metastasis, cancer cells have to degrade a number of natural tissue barriers. These include basement membranes and interstitial connective tissue. The basement membrane is a highly specialised, semi-elastic and continuous extracellular structure that separates organ parenchyma from the surrounding stroma (taken from Ahmad and Hart, 1996). It consists of a number of different proteins and glycoproteins that form a complex cross-linked structure. The most important protein is collagen IV. Other components include laminin, proteoglycans, entactin, and osteonectin (Tryggvason, 1987). The interstitial connective tissue consists of cells distributed in a mesh of collagen fibres (main forms being type

I, II, and III or interstitial collagen), glycoproteins, proteoglycans, and hyaluronic acid.

During metastasis invasive cells traverse basement membranes at least three times (Duffy, 1992): Firstly, during their escape from the primary site through the extra cellular matrix (ECM), secondly, while gaining entry into vessels, and thirdly, while exiting from these vessels at secondary sites. These processes are in part mediated by specific proteolytic enzymes that are produced and released from tumour cells, stromal cells and infiltrating leukocytes. The proteases implicated in degradation of the ECM include serine proteinases, cathepsin B and L (cysteine proteinases), cathepsin D (aspartyl proteinases), and matrix metalloproteinases.

Serine proteinases

Members of this class of endopeptides are characterised by a serine residue at the active site (Duffy, 1993). Some of the members of this group include trypsin, thrombin, plasmin, cathepsin G and plasminogen activators. Plasminogen activators are the best characterised of this group, and they catalyse the conversion of inactive plasminogen to the active plasmin. Plasmin is a broad-spectrum protease and it is responsible for degrading a wide-range of ECM components, such as fibrin, fibronectin, laminin, and certain procollagenases (Testa, 1990). It exists in two forms, tissue-type plasminogen activator (tPA) and urokinase plasminogen activator (uPA), both of which have different biological functions. tPA is thought to be closely connected with intravascular fibrinolysis, whereas uPA is primarily involved in cell-mediated proteolysis during macrophage invasion, wound healing, embryogenesis, invasion and metastasis (Price et al., 1997).

uPA is a glycoprotein initially secreted as a single-chain pro-enzyme. Activation of the pro-enzyme is carried out by limited proteolysis, for example, by plasmin, kallekrein, trypsin, thermolysin, factor XIIa, and the cathepsins (Conese and Blasi, 1995) to produce active uPA. The active form is

a 54 kDa protein consisting of a 24 kDa light chain (amino terminus), and 30 kDa heavy chain. The light chain possesses a uPA receptor binding domain and a single kringle region. The heavy chain contains the catalytic domain, which has homology with other serine proteases. Two catalytic active forms of uPA have been identified of 55 kDa and 33 kDa, the latter consisting of the heavy chain and 21 amino acids of the light chain.

uPA has multiple actions that include converting plasminogen to plasmin, directly activating pro-collagenases, degrading fibrinogen and tissue inhibitors of metalloproteases (DeClerk and Laug, 1996), and is mitogenic to certain cell lines (Kirchheimer et al., 1989). With regard to mitogenesis, it has been shown that uPA can directly activate latent growth factors, such as HGF, and can indirectly activate TGF-β via plasminogen activation (Naldini et al., 1992; Odekon et al., 1994). These activities suggest that uPA has two roles during metastasis, one in furthering ECM degradation, and the other in promoting tumour cell migration and proliferation.

Numerous reports have observed high levels of uPA in tumours and have associated it with an increased risk of tumour recurrence after surgery, for example in bladder cancer (Hasui et al., 1996) and breast cancer (Duffy et al., 1990). Evidence for uPA involvement in tumorigenesis has been shown by blocking uPA function with anticatalytic antibodies and inhibitors. For example, Ossowski et al. (1991) demonstrated in nude mice that human squamous cell carcinoma invasion could be inhibited by anticatalytic antibodies. Retonic acid, an inhibitor of secreted uPA activity, also reduced *in vitro* the invasion of a prostrate carcinoma cell line (Waghray and Webber, 1995; Webber and Waghray, 1995).

In vivo uPA activity is potentiated by binding to its membrane-bound receptor, urokinase plasminogen activator receptor (uPAR; Ellis et al., 1989). The uPAR is a 55-60 kDa highly glycosylated single-chain polypeptide, in which the amino-terminal is involved in uPA binding (Roldan et al., 1990), and is attached to the plasma membrane via a covalent linkage to a glycero-phosphatidylinositol (GPI) moiety. The expression of uPAR is regulated by a

number of motility and growth factors, including TGF-β, EGF and HGF/SF (Lund et al., 1991; Lund et al., 1995; Pepper et al., 1992). Receptor binding concentrates uPA at the cell surface (Moller,1993; Ellis et al., 1992) and accelerates the breakdown of plasminogen, allowing focal digestion of the surrounding matrix. In many experimental systems and human carcinomas it has been shown that increased levels of uPA and uPAR are expressed selectively at the invasive tumour-stroma interface (Skriver et al., 1984; Grondahl-Hansen et al., 1991; Pyke et al., 1991; Pedersen et al., 1994), and are associated with poor prognosis (Duffy et al.,1988; Grondahl-Hansen et al., 1993). Thus, it is clear that the interactions of uPA and uPAR play an important role both directly and indirectly in degrading the ECM and thus promoting invasion and metastasis.

Cathepsins

Cathepsins B and L, characterised by a cysteine residue at their active site, have been increasingly implicated to play a role in cancer progression (Sloane, 1990). Cathepsin B is a 23-28 kDa lysosomal acid hydrolase that has a broad range of endopeptidase activity against myosin, actin, proteoglycans, fibronectin, laminin, the non-helical portion of type IV collagen, certain MMPs, and inactive pro-uPA soluble and receptor-bound forms (Kobayashi et al., 1991). In malignant tissues cathepsin B has some differences, namely, a greater proportion of it is found in association with the plasma membrane and it appears to be more active at neutral and slightly alkaline pH (Keren et al., 1988; Sloane et al., 1990). These properties perhaps allow it to catalyse peptide-bond hydrolysis more efficiently and thus play a greater part in proteolysis during metastasis.

Studies have been performed that suggest involvement of cathepsin B in cancer. It was observed that in patients with advanced squamous cell carcinoma of the uterine cervix, advanced stage carcinoma of the ovary and endometrium, and papillary carcinoma of the thyroid, there was an association

with elevated levels of cathepsin B enzyme activity (Pietras et al., 1979; Shuja and Murnane 1996). Data has also been presented for colorectal carcinoma, where cathepsin B expression correlates with tumour progression and decreased patient longevity (Campo et al., 1994).

Cathepsin L has a similar capacity, like cathepsin B, to efficiently degrade ECM proteins, such as collagen, laminin and elastin, and to strongly activate pro-uPA (Price, 1997). A recent report has shown its activity was strikingly elevated in papillary carcinoma (Shuja and Murnane, 1996).

Cathepsin D, like cathepsins B and L, is also a lysosomal protease. However, unlike cathepsins B and L, it belongs to the aspartyl group of proteases. Initially it is synthesised as a 52 kDa protein and is then transported to the lysosomes, where it is processed to a 48 kDa intermediate form, after which further processing generates two mature forms of 34 and 14 kDa (Rochefort, 1992). In neoplastic cells processing appears to be slower than in normal cells, and as such they accumulate greater amounts of the 52 and 48 kDa forms (Rochefort, 1992). The same author also showed that cathepsin D has mitogenic activity in estrogen-depleted breast carcinoma cells (Rochefort, 1987; Vignon et al., 1986). Recent reports have correlated cathepsin D with tumour aggressiveness in patients with early cervical squamous cell carcinoma (Kristensen et al., 1996). Examples have also been given where capthepsin D expression has a strong prognostic value for poor survival in endometrial cancer (Losch et al., 1996). There are also a number of reports correlating it with metastatic capacity. The most interesting is that of Garcia et al. (1996), in which overexpression of transfected cathepsin D in rat embryo cells increased their malignant phenotype and metastatic potency. It has been postulated that cathepsin D may facilitate metastatic growth by inactivating growth factor inhibitors or by activating the interaction between growth factors and their receptors (Garcia et al., 1996; Jiang et al., 1994a).

Matrix metalloproteinases

The matrix metalloproteinases (MMPs) represent a group of proteases that are involved in ECM degradation. Currently 16 family members have been identified (Table 4), and they share the following properties:

1. A putative zinc-binding domain at the catalytic site.
2. Secretion in an inactive proform (zymogen).
3. Activation by other proteinases.
4. ECM components are their natural substrates.
5. MMP activity is naturally inhibited by tissue inhibitors of metalloproteinases (TIMPs).

There are three major subgroups of MMPs, which are identified by their substrate preferences: collagenases degrade fibrillar collagen, stromelysins degrade proteoglycans and glycoproteins, and gelatinases strongly degrade nonfibrillar and denatured collagens (gelatin). There are also others which do not conveniently fall into these categories. Extensive experimental evidence exists for the involvement of each MMP in tumour progression (reviewed in MacDougall and Matrisian., 1995; Brown, 1993; Ray et al., 1994), from which the following generalisations can be made: i) the number of detectable MMP members tends to increase with tumour progression, ii) the relative level of any expressed MMP tends to increase with increasing tumour stage, and iii) MMPs can be produced by either tumour cells and/or by stromal host cells under the influence of factors from neoplastic cells (Chambers, 1997). This expression pattern of MMPs strongly supports their role in tumour progression. However, one must remember that such correlations between protease levels, tumour growth and metastasis are not universal (Duffy, 1987; Duffy, 1990). Although some studies have found that the transfection of MMP family members has had positive effects on *in vitro* invasion (Matrisian et al., 1991), other studies showed no change in *in vitro* invasion, for example in

Table 4. Main members of the matrix metalloproteinase family.

Group	Enzyme name	Main substrate
Type IV collagenases	MMP-2 (gelatinase A)	Type IV collagen, gelatin collagen types V, VIII, X laminin
	MMP-9 (gelatinase B)	Type IV collagen, gelatin collagen types I, II, IV elastin
Interstitial collagenases	MMP-1 (interstitial collagenase)	collagen types I, II, III, VII, X; gelatins
	MMP-8 (neutrophil collagenase)	collagen types I, II, III
	MMP-13 (collagenase 3)	collagen types I, II, III
Stromelysins	Stromelysin-1 (MMP-3)	proteoglycan, fibronectin, laminin, collagen III, IV, V, IX; elastin, Pro-interstitial collagenase
	Stromelysin-2 (MMP-10)	as for Stromelysin-1
	Stromelysin-3 (MMP-11)	α1-Antitrypsin; laminin and fibronectin (weakly)
	Matrilysin (MMP-7)	proteoglycans, ECM glyco-proteins, collagen IV, gelatins, elastin
Elastases	Metalloelastase (MMP-12)	elastin
	MT1-MMP (MMP-14)	gelatinase A , fibrillar collagens, proteoglycans, ECM glycoproteins
	MT2-MMP (MMP-15)	not determined
	MT3-MMP (MMP-16)	gelatinase A
	MT4-MMP (MMP-17)	not determined
	MMP-18	not determined
	MMP-19	not determined

Adapted from Chambers and Matrisian, (1997); Yu et al. (1997); Ahmad and Hart, (1996).
MT= membrane type.

loss-of-function studies using antisense strategies to downregulate expression of stromelysin (Witty et al., 1994) and matrilysin (Noel et al., 1996). These results raise the possibility that certain proteases are only relevant in a particular tumour model, show that MMP expression does not necessarily

reflect MMP activity, and perhaps indicate that some MMPs affect steps in the metastatic cascade other than extravasation (Chambers and Matrisian, 1997; Duffy, 1992).

MMP activity *in vivo* is regulated by endogenous inhibitors known as TIMPs. To date four TIMPs have been cloned, TIMP-1, TIMP-2, TIMP-3 and TIMP-4, and their amino acid sequences derived (Greene, et al., 1996; Yu et al., 1997). TIMPs inhibit MMPs by forming tight stoichiometric, noncovalent bonds with either the proenzyme or activated enzyme, or by regulating their proteolytic activity (Ahmad and Hart, 1996). TIMPs have been used to provide further evidence that MMPs are required for tumour invasion and metastasis. It has been described that TIMP-1 and TIMP-2 can inhibit ECM degradation by tumour cells and can inhibit the invasion of melanoma and sarcoma cells *in vitro* (Albini et al., 1991; De Clerck et al., 1991), and the lung colonising capacity of B16 melanoma cells *in vivo* (Schultz et al., 1988). Similarly, overexpression of TIMP-1 and TIMP-2 caused a reduction in the frequency of metastasis and in some instances an inhibition in tumour growth in various cancer cell lines (Khokha et al., 1992; De Clerck et al., 1992; Khokha et al., 1994; Tsuchiya et al., 1993; Kawanata et al., 1995). However, it must be recognised that like MMPs, there are several examples where TIMPs have no effect on, or enhance tumour growth and/or metastasis (Solaway et al., 1996; Sun et al., 1996).

Migration

Cell motility is central to the process of tumour metastasis. Local invasion, intravasation, and extravasation of tumour cells all require active cellular motility (Price et al., 1997). Tumour cell motility utilises the machinery of normal cellular motility but in an aberrant manner, allowing the migration of tumour cells to regions that constitute abnormal spread. It has been found that tumour cells respond in a motile fashion to a variety of substances, such as host-derived motility and growth factors, ECM components and tumour

secreted factors (Table 5; Kantor and Zetter, 1996; Levine et al., 1995). Cell motility is governed by the nature of the motile response and source of stimulation, and can be defined as: i) chemotactic: a directional cellular migration in response to a positive gradient of a soluble factor, e.g., ECM fragments or growth factors; ii) chemokinetic: a stimulation of directed motility, resulting in random cellular migration away from the original site, e.g., hepatocyte growth factor/scatter factor (HGF/SF, Weidner et al., 1993) and autocrine motility factor (AMF, Liotta et al., 1986; Silletti et al., 1991); and iii) haptotactic: directed cellular motility in response to a positive concentration gradient of immobilised attractant, e.g., immobilised attractants from the ECM (Brandley and Schnaar, 1989). Tumour cell motility results

Table 5. Examples of molecules that control growth and motility.

Motility stimulation and growth stimulation	PDGF-AA and BB TGF-beta EGF/TGF-alpha basic FGF acidic FGF IGF-1 IGF-2 ✡ PD-ECGF G-CSF/GM-CSF
Motility stimulation and growth inhibition	TGF-beta IL-1 ✠ IL-3 ✠ IL-6 EGF TNF-alpha
Motility inhibition and growth inhibition	TGF-beta TNF-alpha INF-gamma
Motility	AMF ATX SF/HGF MSF

Adapted from Duffy, (1996).
ATX Autotaxin
IGF insulin-like growth factor
MSF migration-stimulating factor
✠ Stoker et al. (1991); Jiang et al. (1994b).
✡ Negus et al. (1996).

from these stimuli, which are derived from the local cellular environment and from from autocrine and paracrine secretions. I will consider some of the best described motility factors.

AMF is a cytokine that can induce chemokinesis and chemotaxis in tumour cells (Liotta et al., 1986). It leads to the production of pseudopodia which are necessary for cellular locomotion (Guirguis et al., 1987). Its effects in normal tissues has been observed during such processes as wound healing and embryogenesis. There has been intense study into defining what role it may have in conferring invasive and metastatic capabilities on neoplastic cells. These studies postulate that AMF plays a role in tumour metastasis (Van Roy and Mareel, 1992). It has been shown that AMF is a marker of transitional cell carcinoma of the bladder when present in the urine of these patients (Guirguis et al., 1988). Expression of the AMF receptor gp78 correlates with a malignant potential in bladder cell lines (Silletti et al., 1993) and with metastatic potential in human bladder cancer specimens (Otto et al., 1994).

HGF/SF is both an autocrine and paracrine growth factor that stimulates the motility of epithelial and endothelial cells (Stoker, 1989; Stoker and Gherardi, 1989; Stoker et al., 1989). It induces the scatter or chemokinetic locomotion of epithelial colonies, thus inducing an invasive phenotype. Its effects on the cell growth, morphology and motility in many normal cells and tumour cell lines have been extensively studied (Bhargava et al., 1993; Moriyama et al., 1996; Nusrat et al., 1994). HGF/SF-induced invasive growth is mediated by the *Met* receptor, a member of the tyrosine kinase receptor family (RTKs). In human tumours, mounting evidence from expression studies has suggested a role for *Met* in carcinomas derived from the follicular epithelium (Di Renzo et al., 1994; Di Renzo et al., 1992), in ovarian carcinomas and pancreatic cancers (Di Renzo et al., 1995) and in breast carcinoma (Jin et al., 1997). In these studies, protein over-expression was notably higher in the metastases of the primary carcinomas examined. In an experimental setting, NIH 3T3 cells endogenously producing HGF/SF were transfected with murine *Met* , causing the cells to become highly tumorigenic

and metastatic, due to the completion of an autocrine loop (Jin et al., 1996; Rong et al., 1992; Rong et al., 1994). These results suggest the combination of HGF/SF and its *Met* receptor play an important part in tumour progression by stimulating cancer cell growth and motility.

Epidermal growth factor (EGF) is known to be a potent mitogen for normal cells and tumour cells. In addition to its proliferative effects, it has also been observed to induce motility in a number of tumour cells, for instance in primary gliomas (Engebraaten et al., 1993), squamous carcinoma cells (Shibata et al., 1996), and renal carcinoma cells (Price et al., 1996). EGF is the ligand for epidermal growth factor receptor (EGFR), a RTK. It is believed that through their interaction motility is induced, for example by the formation of lamellipodia in metastatic mammary carcinoma (Segall et al., 1996). Coexpression of both EGF and EGFR has been shown to be of prognostic significance and to play a possible role in the pathogenesis of a number of human cancers (Tateishi et al., 1990; Gorgoulis et al., 1992). For instance, it has been observed that over-expression of EGFR in bladder cancer (Neal et al., 1990), breast cancer (Nicholson et al., 1991; Klijn et al., 1992; Koenders et al., 1993), and glioblastoma multiforme (Hiesiger et al., 1993) coincides with a poor prognosis.

It has been found that another member of the RTKs, the proto-oncogenic receptor ErbB-2 is overexpressed and correlated with poor prognosis for breast carcinoma patients (Hynes and Stern, 1994). ErbB-2 is bound preferentially by other ligand-filled RTKs receptors such as EGFR to form heterodimers. These heterodimers are characterised by an extremely high signal potency, due to a reduced rate of ligand dissociation and efficient signalling of ErbB-2 through MAP-kinases, thus enhancing mitogenic activity (reviewed in Alroy and Yarden, 1997). The interaction of EGF and EGFR can induce motility in cancer cells, and perhaps further interaction with ErbB-2 could provide enhanced motility and metastasis formation. Additionally, this interplay of factors with ErbB-2 passes on a selective advantage to tumour cells

by better utilising the EGF-like growth factors bound to other EGFR receptors.

Insulin-like growth factors (IGFs) have been observed to stimulate chemotactic responses in tumour cells. These act through the type I IGF receptor. Stracke et al. (1988) showed that IGF-I stimulated motility in a human melanoma cell line. Similarly, Kohn et al. (1990) used IGF-I and II to stimulate melanoma cell motility in Boyden chamber assays. These factors could facilitate tumour cell invasion and growth at metastatic sites.

The migration-stimulating factor (MSF) is not classified as a growth factor or cytokine. It is a 190 kDa secreted protein produced by human foetal fibroblasts (Schor, 1988). Schor et al. (1989; 1990) have shown that this factor can induce motility in fibroblasts, is detected in the sera from breast cancer patients and stimulates hyaluronic acid secretion. However the complete mechanism of its action with regard to motility is unclear, and as such requires further investigation.

Chemotactic and chemokinetic properties have been described for TGF-β in different cell lines (Wright et al., 1993). Its effects have been attributed to pseudopodia formation, membrane ruffling, and the regulation of fibronectin and hyaluronan production to promote cell locomotion. Furthermore its effects on tumour progression may also be through the stimulation of protease production that may activate other growth factors and ECM molecules (Stoker and Gherardi, 1991; Ellis and Schor, 1996; Turley et al., 1991; Agarwal et al., 1994; Samuel et al., 1992; Welch et al., 1990).

Other cytokines of interest that have been shown to stimulate motility in endothelial and tumour cells are platelet-derived growth factor (PDGF; Pauly et al., 1977), fibroblast growth factors (FGFs; Gospodarowicz, 1990) and tumour necrosis factor α (TNF-α; Rosen et al., 1991). Apart from facilitating cellular motility these factors have the overlapping function of inducing endothelial cell migration, which is an important process of the formation of new blood vessels in tumours (Negus and Balkwill, 1996).

Tumours and angiogenesis

Metastatic cancer cells utilise the processes of invasion, proteolysis and migration to colonise other tissue regions and organs. However, in order for further growth of these cells to occur at these metastatic sites, the metastatic cells must neovascularise. This occurs by angiogenesis, the development of new blood vessels, which is important in normal biological processes such as placental, embryonic, foetal and post-natal growth, wound healing and cyclically in the ovarian follicle, corpus luteum and post-menstrual endometrium (Folkman, 1971; Christenson et al., 1996; Modlich et al., 1996; Risau., 1995; Risau., 1996). Without angiogenesis, metastatic cells fail to form tumours beyond 2 mm diameter (Folkman, 1989; 1990; 1995). The initiation of angiogenesis is thought to be regulated by insufficient vascular supply, and the resulting hypoxia leads to a feedback response of neovascularisation (Stein et al., 1995). There is strong evidence to suggest that this feedback response is principally mediated by vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) (Aiello et al., 1994; Banai et al., 1994; Miller et al., 1994; Minchenko et al., 1994; Pe'er et al., 1995; Shweiki et al., 1992). VEGF/VPF is potent in inducing new blood vessel formation (Senger et al., 1993). In addition to its mitogenic activity VEGF/VPF is a strong promoter of microvascular permeability (Senger, 1990), causing the extravasation of plasma proteins (Kondo et al., 1993). Recent studies have documented the expression of VEGF/VPF in a correlation with vascularity, metastasis and proliferation in human and animal cancers (reviewed in Claffey and Robinson, 1996). In addition to these reports, experiments designed to inhibit VEGF/VPF activity *in vivo* have demonstrated a reduction in tumour growth and angiogenesis (Kim et al., 1993; Millauer et al., 1994). Once new blood vessel growth is obtained, further angiogenic interactions occur between the host and tumour, that are mediated by other cytokines such as FGFs, PD-ECGF and TNF-α leading to increased vascularisation, tumour size and metastatic growth (reviewed in Norrby, 1997).

The recent work of O`Reilly et al. (1993; 1994; 1996; 1997) highlights that there are other molecules produced by tumours that can dramatically influence angiogenesis in tumour growth and metastasis. They observed in certain tumour types that removal of the primary tumour results in rapid growth of the metastases, suggesting that the primary tumour is responsible for stimulating angiogenesis in its own vascular bed, and inhibiting angiogenesis in the vascular bed of its metastases (O`Reilly et al., 1993). They hypothesised that the primary tumour stimulates its angiogenesis by producing angiogenic stimulator(s) in excess of angiogenic inhibitor(s). However, the angiogenic inhibitors(s) due to their longer circulatory-half-life, reach the vascular bed of the metastases in excess of the angiogenic stimulator(s) produced by the primary tumour or the metastases themselves, resulting in the inhibition of metastatic growth (O´Reilly et al., 1994). Removal of the primary tumour thus results in the loss of angiogenic inhibitors and greater growth of the metastatic sites due to increased neovascularisation. They found two tumour models that satisfied this hypothesis, a variant of lewis lung carcinoma (O´Reilly et al., 1994) and murine haemangioendothelioma (O´Reilly et al., 1997), and isolated the angiogenic inhibitors angiostatin and endostatin from these systems, respectively. The administration of these angiogenic inhibitors could potently block endothelial cell proliferation, and tumour and metastatic growth. Recently they also used angiostatin to induce and sustain dormancy of human primary tumours in mice (O´Reilly et al., 1996), suggesting that this molecule and other inhibitors produced by tumours could have a potent role in treating cancer. Clinical trials with these agents is currently underway. From these observations it can be concluded that tumour cell proliferation at primary and metastatic sites is angiogenesis-dependent.

Other tumour and metastasis associated antigens

Aberrant glycosylation

Specific glycosphingolipids (GSLs) have been shown to be associated with oncogenic transformation (Hakomoro et al., 1968; Mora et al., 1969). On some tumours they are more highly expressed than on normal tissues, causing a humoral response, thus allowing them to be described as tumour-associated antigens. A number of "typical tumour-associated GSL antigens" have been described, including Globo series (Gb3, Globo H and Disialosylgalactosyl-globoside), Ganglio series (GM3, GD3, GD2 and Fucosyl-GM1), and the Lacto series (Le^x, Le^x-Le^x, Le^y-Le^x, Le^a-Le^a, Sle^x, Sle^x-Le^x and Sle^a; Hakomori et al., 1997).

Nearly all cancers show great differences in their GSL composition and metabolism compared to parental normal cells (Hakomari et al., 1997). It is believed that GSL clusters enhance tumour cell motility and invasiveness through interactions with some unknown ligand(s), GSLs on other cells, the indirect modulation of other adhesion molecules, and the triggering of transmembrane signalling (Hakomori, 1997). Only a few cases of such interactions have been described. For example, the GSL GM3 is upregulated on the most invasive and metastatic variant cell line of the mouse melanoma B16. Here it can act as an adhesion molecule for two other GSLs, Gg3Cer and LacCer that are expressed on non-activated vascular endothelial cells (Kojima et al., 1992). Metastasis in this model was blocked by antibodies to GM3 or Gg3, or by the administration of liposomes containing GM3 or Gg3 (Otsuji et al., 1995). In another system it was shown that the GSL disialosyl-galactosylgloboside is upregulated in human renal cell carcinoma (Saito et al., 1997) that extensively metastasises to the lung. In cell lines derived from the carcinoma, the expression pattern remained, and the binding of these cells to perialveolar lung tissue sections was inhibited by an antibody directed against disialosylgalactosylgloboside (Satoh et al., 1996), suggesting the presence of an

undetermined ligand for disialosylgalactosylgloboside that may mediate metastasis.

Lewis antigen-selectin interactions

Changes in N-linked and O-linked glycosylation of glycoproteins have been observed to be involved in the metastatic process. Studies have shown the existence of a correlation between abnormal glycosylation of human cancers and the invasive/metastatic properties of certain tumours (Hakomori, 1996). For example, it has been shown that carcinoma cells highly express the carbohydrate sialyl Le^a (Magnani et al., 1982) and that carcinoma and leukemic cells express sialyl Le^x (Fukushima et al., 1984). It has also been observed that colon carcinoma patients presenting with upregulated sialyl Le^x structures on their tumour cells have a poorer prognosis than those presenting with lower expression of sialyl Le^x (Nakamori et al., 1993).

One way in which changed glycosylation patterns can contribute to metastasis is during intravasation and extravasation as sialyl Le^a and sialyl Le^x, as both are ligands for selectins which are present on endothelial cells (Berg et al., 1991; Takada et al., 1991; Renkonen et al., 1997). For example, some tumour cells express increased levels of Le^x and Le^a, and highly metastatic colonic carcinoma cells expressing such increased levels bind more readily to E-selectin on activated human endothelial cells than do related cell lines of lower metastatic potential (Fukuda, 1996). It has been shown that antibodies to E-selectin or synthetic Le^x inhibited tumour cell attachment to activated endothelial cells, signifying that in metastasis formation, the interaction of endothelial-expressed E-selectin with tumour cells expressing Le^x could be a critical factor in some cancers (Iwai et al., 1993). P-selectin is also expressed on the endothelium, and has been implicated in tumour cell arrest in microvessels by mediating the binding of platelets to specific tumour cells, and the formation of tumour cell clumps and aggregates in the circulation (Stone and Wagner, 1993). These studies and others provide evidence that selectin-

ligand interactions could be responsible for the adhesion of metastatic cells to the endothelium, and are as such important in the initial events leading to tumour cell intravasation and ultimately metastasis formation.

Mucins

During malignant transformation of epithelial cells, membrane expression of high molecule weight proteins known as mucins often changes. In normal physiological states they are produced by various secretory epithelial cells such as the mammary gland, salivary glands, digestive tract, respiratory tract, kidney, prostate, urinary bladder, uterus and rete testis (Zotter et al., 1988). Structurally they are glycoproteins containing negatively charged O-linked oligosaccharides, which accounts for more that two-thirds of the proteins molecular weight, and provides an extraordinary degree of microheterogeneity (Shimamoto et al., 1989). Malignant cells upregulate mucin production, resulting in large amounts of the protein being shed or secreted, and change the glycosylation of the protein backbone which generates new epitopes or exposes the polypeptide core (Devine and Mckenzie, 1992). These alterations in protein expression contribute to changes in cancer cell proliferation, immune recognition, and cellular adhesion, which have been shown to influence the invasive and metastatic properties of malignant cells (Ho et al., 1995). A number of studies of cancers of the lung (Yu et al., 1996), stomach (Ho et al., 1995a), colon (Schwartz et al., 1992) and pancreas (Ho et al., 1995b) have identified changes in mucin expression that are associated with cancer progression and metastasis formation. The best described is that of colon cancer.

It had been observed that patients presenting with mucinous colon cancer often had advanced disease and a poor prognosis (Schwartz et al., 1992). Bresalier et al. (1991) demonstrated that increased mucin production by colon carcinoma cells correlated with their metastatic potential. They found that high mucin producing (HMP) colon cancer cells adhered better to a

reconstituted basement-membrane gel (matrigel) than a low mucin producing (LMP) colon cancer cell line. The addition of purified colon cancer mucin to LMP parental cell line enhanced the adhesion of these cells to laminin and matrigel. This supports the hypothesis that colon cancer cell mucins play a role in heterotypic interactions between tumour cells and the ECM. Schwartz et al. (1992) used benzyl-α-N-acetylgalactosamine to inhibit the production of fully glycosylated mucin from HMP cells. These authors observed that treated HMP colon cancer cells had reduced invasive ability as compared to non-treated cells. These studies provide evidence that mucin-type glycoproteins affect various stages of colon-cancer metastasis. However, the nature of mucin-ECM interactions remains to be elucidated.

Mucins are modified by a number of carbohydrate antigens, including Le^a and Le^x, and mucin core region antigens, such as T and Tn (Matsushita et al., 1991; Yamori et al., 1989; Itzkowitz et al., 1988). Yoon et al. (1996) have shown that E-selectin binds to Le^a and Le^x expressed on mucins from HM7 human colon cancer cells. They observed that cells treated *in vitro* with benzyl-α-N-acetylgalactosamine exhibited reduced expression of surface Le^a and Le^x and binding to E-selectin, suggesting that surface mucin on colon cancer cells is critical for their attachment to the endothelium and the generation of metastasis.

5T4 antigen

Comparisons between the growth and invasive properties of embryonic tissues and tumour cells have stimulated the search to find functional similarities in cell surface molecules (Myers et al., 1994). A tissue that has been considered is the trophoblast, the foetal tissue of the placenta. It exhibits regulated invasion of the uterus while avoiding the maternal immune system. Thus antigens present on the trophoblast may function to facilitate the growth and survival of the foetus as a semi-allograft *in utero*, and similarly function to promote tumour invasion (Myers et al., 1994).

The 5T4 oncofoetal antigen was determined by the 5T4 monoclonal antibody, raised against human placental trophoblast (Hole and Stern, 1988). Structurally, the antigen is a 72 kDa transmembrane glycoprotein, consisting of a 310 amino acid extracellular domain that is heavily glycosylated (Hole and Stern, 1990) and contains leucine-rich repeats, and a 44 amino acid cytoplasmic domain (Myers et al., 1994). Immunohistochemical studies revealed that the antigen is strongly expressed on foetal trophoblast membranes but is absent from most normal non-pregnant tissue, with only a few specialised epithelia being weakly positive. In contrast, it is expressed by a variety of transformed embryonic and carcinoma-derived cell lines and by many human carcinomas (Southall et al., 1990; Hole and Stern, 1988). Studies of this type have also revealed a strong correlation between 5T4 histochemical staining in ovarian, colorectal and gastric carcinomas and metastasis (Starzynska et al., 1992; Wrigley et al., 1995), suggesting a strong connection between 5T4 expression by tumours, disease progression, metastasis and patient survival. Recent work by Carsberg et al. (1996) has supported this correlation. In this study the authors found that transfection of full-length 5T4 cDNA into epithelial cells alters cell-cell contacts and cell motility. In transfected CL-SI murine mammary cells, 5T4 expression was associated with dendritic morphology, a reduction in actin/cadherin-containing contacts and increased motility. Transfection with a 5T4 truncated-cytoplasmic-tail mutant form resulted in transfectants with a reduction in actin-cadherin-containing contacts but motility was unchanged. These observations suggest that 5T4 can influence cellular characteristics through both its intracellular and extracellular domains, reflecting a role for 5T4 in the invasion process (Carsberg et al., 1996).

Conclusion

I have presented here an overview of the major groups of molecules involved with metastasis formation. Many more of these molecules are certain to be added to the growing list. Tumour cells take advantage of the normal

physiological properties of these molecules, and due to differences in quantitative and/or qualitative properties in these molecules, are able to metastasise and proliferate at other tissue sites. These molecules can function in multiple ways in metastasis during the different steps of proteolysis, adhesion and migration. For example, integrins primarily mediate cell-ECM adhesion indicating that they are involved in tumour-cell adhesion and migration. They can also bind MMPs and localise MMP activity during cellular migration at the tumours perimeter, signifying that integrins can also be important in proteolysis. Active uPA plays a direct role in degrading the ECM. In addition, it has been shown to activate directly HGF/SF and indirectly TGF-β (Naldini et al., 1992; Odekon et al., 1994). These activities of uPA suggests that it not only modulates ECM degradation, but also tumour cell migration and proliferation. It is through this plethora of molecules and their varied activities that tumour cells complete the metastatic cascade.

Aims and experimental system

The realisation that many molecules expressed on tumour cells are involved in multiple steps during the formation of metastases has advanced our understanding of the cellular and molecular mechanisms of cancer. Thus, further research into molecules specifically expressed or lost on metastatic tumour cells can only lead in the end to a better clinical outcome for patients and ultimately disease prevention.

Many surface molecules associated with cancer have been identified by immunological procedures to generate monoclonal antibodies specific for tumour cells (Brooks et al., 1993). The traditional approach is to directly immunise mice with the intact metastatic tumour cell. However, this procedure can prove to be inefficient, for tumour cells present an array of molecules that can be immunodominant and perhaps play no functional role in metastasis. To overcome these inefficiencies an experimental technique known as subtractive immunisation (Byrne and Cox, 1986; Williams, 1992) has been successfully

used to enrich the immune response against antigens that are involved in metastasis (Brooks et al., 1993; Sleeman et al., 1998). It reduces the immunodominancy of non-metastasis-associated molecules by an immuno-depressive procedure, and thus achieves a stronger immunological response against metastasis-associated-antigens. My aim was to utilise this procedure to isolate, identify and characterise metastasis-specific surface antigens expressed on clones from the BSp73AS pancreatic carcinoma cell system which have defined metastatic properties (Matzku et al., 1983).

MATERIALS AND METHODS

MATERIALS

Chemicals. All general chemicals were purchased from *Carl Roth GmbH + Co.*, Karlsruhe, *Merck*, Darmstadt or *Sigma Chemie GmbH*, Deisenhofen, and were of the highest quality. Radiochemicals were obtained from Amersham.

Antibodies. All antibodies other than those made by myself were obtained from Jonathan Sleeman.

Cell culture and manipulation

Cell culture. All cells were maintained in a 37°C incubator (Forma Scientific, Labotect GmbH, Göttingen) with 6% CO_2. Tissue culture media was made from powder obtained from *Gibco* and reconstituted according to the manufacture`s instructions. All medium was supplemented with 100U/ml penicillin and 100µg/ml streptomycin. Trypsin was obtained from Difco Laboratories (Detroit, USA) and was diluted to 0.25% in 15mM sodium citrate, 134mM potassium chloride. Culture conditions, passaging procedures and harvesting were by established methods (e.g. Freshney, 1986).

Cell lines and medium The derivatives of the Bsp73 pancreatic tumour cell lines (BSp73ASML [ASML], and BSp73AS clones 0AS, 1AS, 3AS, 7AS, 10AS [Matzku et al., 1983]) were maintained in RPMI 1640 medium containing 10% FCS. The AS transfectants ASpSV14 (transfected with CD44v4-v7 [Günthert et al., 1991]) were maintained in RPMI 1640 medium containing 10% FCS, supplemented with 300µg/ml G418. AT-1, AT-2.1, AT-3.1, AT-6.1, MatLyLusu, G and MatLu cell lines were cultured in RPMI 1640 medium containing 10% FCS and 250nM dexamethasone (Isaacs et al., 1978; Isaacs et al., 1986; Ichikawa et al., 1992; Ichikawa et al., 1991). CREF, CREF-T24,

MT450, MTPa, MTC, MTLN2, MTLN3, MTLy and BDX2 cells were maintained in Dulbecco's modified Eagle's medium containing 10% FCS (Hofmann et al., 1993; Dunnington et al., 1984; Lichtner et al., 1987; Neri et al., 1981; Sleeman et al., 1996). SP2/0 cells were cultured in RPMI with 10% FCS (Köhler and Milstein, 1976). In experiments in which tunicamycin was used, tunicamycin was added to the medium of growing cells to a final concentration of 7.5µg/ml for the indicated time.

Frozen stocks. Cell stocks were maintained in liquid nitrogen. Logarithmically growing cells were trypsinised, centrifuged at 250×g and resuspended in 1ml freezing mix (90% FCS and 10% DMSO, Fluka Chemie AG, Buchs, Switzerland). The freezing vials containing the cells were placed in a rack, after which a towel was wrapped around the rack and they were placed in a -80°C freezer. They were then transferred to liquid nitrogen the next day. To return the cells to culture, the vials were thawed at 37°C, pipetted into 10ml medium, centrifuged at 250×g and plated out in fresh medium.

METHODS

Protein methods

Cell Lysates. Cells were grown to confluency and then removed from the petri dish by physical scraping using a rubber policeman, or by incubation with PBS (Mg^{2+} and Ca^{2+} free) containing 5mM EDTA. The cells were counted with a Coulter counter and resuspended to a final concentration of 1×10^7 cells/ml in sample buffer (125mM Tris.HCl pH 6.8, 2% SDS) containing 10% glycerol for non-reducing, and 10% β-mercaptoethanol or 100mM dithiothreitol (DTT) for reducing sample buffer. Samples were boiled for five minutes and then sonicated for 15 to 20s to break down chromosomal DNA. 5×10^5 equivalent cell volumes were loaded into each gel slot.

SDS-PAGE. Proteins were separated electrophoretically on the basis of size using the methods of Laemmli (1970). The resolving gel contained 10% acrylamide, unless otherwise stated. Samples were run into the stacking gel at 50V, and then run at 30V overnight, or 125V during the day. Gels which were Coomassie stained were incubated with Coomassie (0.25% Coomassie brilliant blue, R-250, 50% methanol and 10% acetic acid) for 4-24h. Gels were destained in 10% acetic acid, 10% methanol for 24hr or more.

Immunoprecipitations. For immunoprecipitations of cell lysates, cells were grown to 80-100% confluency, washed with cold PBS, counted by a Coulter Counter and resuspended at 1×10^7 cell/ml in ice cold RIPA buffer (150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris, pH 7.5) containing 2mM PMSF. The DNA was sheared by passing the lysate through a needle a few times, and the non-soluble material pelleted in a 4°C bench microfuge at 14,000 RPM. To reduce the background, the supernatant was taken and incubated with a final concentration of 1% globulin free BSA and also precleared with a 1/10 volume of protein-G agarose beads. The mixture was then rotated end over end for 1-2 hours at 4°C, after which the beads were spun out and the supernatant used for immunoprecipitations. For immuno-precipitations of conditioned tissue culture medium, cells were grown overnight in a minimal volume of medium. The conditioned medium was collected, centrifuged to remove cell debri and extensively dialysed against PBS.

Aliquots of the prepared lysates corresponding to 5×10^5 cells were incubated with 5µg of monoclonal antibody (spun in a microfuge to remove any precipitate) and 50µl 50% RIPA slurry of protein G agarose at 4°C for 2-24 hours. The protein G beads were pelleted by a 30 second spin in a microfuge, the supernatant was discarded and 1ml of RIPA was added. The beads were resuspended, then spun out as before and the supernatant was aspirated off. The beads were washed this way in RIPA buffer four times. After the final wash, the beads were spun down briefly in a microfuge, and a

Hamilton syringe used to remove all of the remaining RIPA from the beads. The beads were resuspended in 50µl sample buffer, boiled, and the supernatant subjected to SDS-PAGE and western blotting.

Western blotting. Proteins separated by SDS-PAGE were electrically transferred to nitrocellulose (Schleicher and Schuell, Dassel, Germany) by the method of Towbin et al. (1979). Blots to be cut into strips were made from gels with large loading slots, with 5×10^5 cell equivalents of protein electrophoresed per cm of slot. Filters were washed in TBS (25 mM Tris.HCl, pH 8.1, 150 mM NaCl) for 10 minutes, then used or stored frozen at -20 °C. Blots were blocked in milk buffer (4% milk powder and 0.5% Tween 20 in TBS) for 30 minutes, then probed with the first antibody in milk buffer for 1-2 hours. After washing three times in milk buffer for two minutes each wash, the blot was incubated in milk buffer for one hour with an appropriate antibody coupled to horse radish peroxidase to allow Enhanced Chemi-Luminescence (ECL, Amersham) detection. The blot was washed three times for two minutes each wash, and once for 10 minutes in milk buffer, then 4 times for 2 minutes each wash in TBS. ECL detection was performed according to the manufacturer's instructions.

Glycosylation Studies by enzyme digestion of immunoprecipitates. Enzyme digestions were completed while the antigen was attached to protein G beads after immunoprecipitation, and a final wash into phosphate buffer pH 7.0. For N-glycosidase F and O-glycosidase digestion, 5 µl 1% SDS/20mM phosphate pH 7.0 was added to the final washed immunoprecipitates. After boiling for 5 minutes, and cooling on ice for 2 minutes, 45µl of 20mM phosphate pH 7.0 containing 0.5U of N-glycosidase F (Boehringer Mannheim) or 2.5mU O-glycosidase (Boehringer Mannheim) was added to the immunoprecipitates. For neuraminidase digestion, the immunoprecipitate was mixed with 50µl 50mM sodium acetate pH 5.5, 4mM $CaCl_2$, 0.1mg/ml BSA and 5mU neuraminidase (Boehringer Mannheim). The enzyme digestions were

incubated at 37°C for 3 hours, then the supernatant was analysed by SDS-PAGE and western blot.

Antibody production and purification

Cell ELISA. Target cells were harvested using PBS/5mM EDTA and resuspended at 2×10^6/ml in RPMI/10% FCS. 50µl of test antibody solutions were pipetted into wells of U form 96 well plates, and 50µl aliquots of cells were added as appropriate. The cell/antibody mix was incubated for 3 hours at 37°C, then washed three times with 200µl aliquots of PBS. Bound primary antibody was detected with rat-anti-mouse antibody coupled to horse radish peroxidase, followed by ABTS [2, 2´azino-di-(3-ethyl-benzthiazolin-sulphonate)] treatment (Harlow and Lane, 1988).

Subtractive immunisation. Tumours were surgically removed and dissociated by physical force through a cell dissociation sieve. The tumour dissociate was applied to a 40µM cell sieve (Falcon), and the isolated tumour cells washed three times in PBS. Tissue culture cells were harvested with PBS/5mM EDTA, then washed three times in PBS. Pre-bleeds were taken and male balbC/BL6 F1 hybrid mice were injected intradermally with 2×10^6 cells (the tolerogen). After 24 and 48 hours, 200mg/kg cyclophosphamide was injected intraperitoneal (i.p.) into each mouse (King et al., 1988; Many et al., 1970; Prigozhiuna et al., 1980; Sensenbrenner et al., 1979; Williams et al., 1992).. Three weeks later, the mice were similarly injected with tolerogen cells and cyclophosphamide. Ten days later, test bleeds were taken. Three weeks after the last injection with tolerogen cells, 2×10^6 immuogen cells were injected i.p.. Immunogen cells were injected twice more, with three week intervals between each injection. Ten days after the last immunisation, test bleeds were taken. The pre-bleeds were compared together with test-bleeds in cell ELISAs using tolerogen and immunogen cells as targets to identify mice in which the immune response to tolerogen cells was maximally destroyed and in which the

immune response to immunogen cells was maximally enhanced. These mice were selected for hybridoma production and were allowed to rest for 4 weeks before fusions were performed.

Monoclonal antibodies. Murine hybridomas were made using the 30% PEG spinning method (Harlow and Lane, 1988). SP2/0 myeloma cells were fused to immune spleen cells from two mice immunised by subtractive immunisation and boosted 5 days before the fusion by injection with 2×10^6 immunogen cells i.p.. Hybridomas were screened by using tolerogen and immunogen cells as targets in cell ELISA assays. Hybridomas recognising only the immunogen cells were grown up into 10ml cultures and frozen in liquid nitrogen. Selected hybridomas were thawed, and cloned by limiting dilution (Harlow and Lane, 1988) over several rounds until all the wells containing single colonies were positive for specific antibody production. The monoclonal antibodies were isotyped by ELISA using ISOStrip™ obtained from Boehringer Mannheim.

Purification of immunoglobulins using Protein G. Supernatant from hybridomas was collected and centrifuged at 4-5 K for 10 minutes to remove cellular debri. Antibodies were precipitated by the addition of ammonium sulphate to a final concentration of 50%. The solution was incubated overnight at 4°C with gentle stirring. The precipitate was pelleted by centrigugation at 5-6 K for 15 minutes. The pellet was resuspended in 10-20ml 150mM NaCl, 20mM phosphate pH 7.0 and dialysed for several hours against the same buffer. After dialysis, the protein solution was centrifuged at 10 K for 10 minutes and the resulting supernatant added to 2ml of a 50% slurry of Protein G plus agarose (Dianova) for each 50ml of supernatant. The mixture was incubated for 2 hours or over-night at 4°C with rotation. After incubation, the beads were centrifuged at 3 K for 10 minutes and the supernatant discarded. The beads were then washed 4 times with 20mM phosphate pH 7.0, 150 mM NaCl. After the final wash, the beads were taken up in 10ml of wash buffer and transfered to an Econopak column and the excess fluid allowed to drain

off. The antibodies were eluted with 100mM glycine pH 2.5 and ten 0.5ml fractions collected. Each aliquot was neutralised by adding 50µl 1M Tris pH 9.6. A further 8 fractions was collected using 0.5ml aliquots of 100mM sodium bicarbonate pH 10.8. Each aliquot was neutralised by the addition of 87µl 1M sodium citrate pH 5.0. Protein concentration was estimated from the O.D.280 (Harlow and Lane, 1988), and the fractions containing significant amounts of antibodies were pooled and dialysed against PBS. The purified antibodies were stored in aliquots at -20°C.

Immunological methods

Preparation of Protein G-Antibody affinity columns-direct coupling. 2 mg of antibodies in PBS were mixed with 1 ml of protein G beads by gentle rocking for 1 hour at room temperature. The beads were then washed twice with 10 volumes of 0.2M sodium borate pH 9.0. The beads were then resuspended in 10 volumes of 0.2M sodium borate pH 9.0 containing Dimethylpimelimidate (Pierce) at a final concentration of 20mM and mixed on a rocker for 30 min. The reaction was stopped by washing the beads once in 0.2M ethanolamine pH 8.0. The beads were then incubated for 2 hours at room temperature in 0.2 M ethanolamine pH 8.0 with gentle mixing. After the final wash the beads were resuspended in PBS containing 0.01% merthiolate. To check the efficiency of coupling of the antibodies to the beads, 10µl equivalents of beads were removed before and after the addition of dimethylpimelimidate and boiled in reducing sample buffer. The samples were run out on a 10% SDS-PAGE gel and stained with Coomassie blue. Good coupling was indicated by the presence of heavy-chain bands (55 KDa) in the "before" but not in the "after" lanes.

Immunohistochemistry. Rats were subcutaneously injected with 1AS, 10AS and ASpSV14 tumour cell lines. After 3 to 4 weeks the animals were killed and the tumours removed. For paraffin sections the tumours were fixed in 4% paraformaldehyde and then embeded in paraffin wax. Sections (7µm) were de-

waxed by two 5 minute incubations in Xylene and then rehydrated through reducing 100, 95, 90, 80, 70 and 50% concentrations of ethanol. After washing in PBS for 10 minutes, the sections were pre-incubated with normal goat serum (10% in PBS). For frozen sections, the tumours were removed, cut into $1cm^3$ cubes and frozen on dry ice in a pre-cast metal mould immersed in Freezing gel (Jung, Tissue Freezing medium). The sections were cut (7μm), fixed in ice-cold methanol (5 min) followed by ice-cold acetone (1 min), washed in PBS and pre-incubated with normal goat serum (10% in PBS). Both paraffin and frozen sections were then treated as follows. After washing 3 times with PBS, sections were incubated for 1 hour with primary antibody (5-10μg/ml, in PBS/10% FCS). Endogenous peroxidase activity was then blocked by incubating the sections in 0.3% H_2O_2 in methanol for 15 min. Secondary biotinylated antibody (anti-mouse $F(ab')_2$, DAKO, Santa Barbara, CA) was subsequently added for 30 min, followed by horseradish-peroxidase coupled to biotin as a streptavidin-biotin-peroxidase-complex (DAKO) for another 30 min. The immune complex was visualised colorimetrically by incubation with 3,3-amino-9-ethyl carbazole (SIGMA) for 10 min. Colour development was then stopped in H_20. Cell nuclei were counter-stained with hematoxylin and the sections mounted with glycerine-gelatin and viewed by microscopy.

For immunofluorescence studies, adherent cells were grown on 8 well chamber slides (Nunc) and suspension cultures were affixed to silane treated slides using a Cytospin centrifuge (Shandon Southern). In both cases, cells on slides were washed 3 times with PBS, fixed in 4% paraformaldehyde, then incubated for 30 minutes in PBS/10% FCS (FPBS). Antibody solution was then added in FPBS and incubation continued for 2 hours. The cells were washed 3 times with PBS, then incubated for 1 hour with Rhodamine-conjugated affinipure goat anti-mouse Ig (Jackson laboratories) diluted in FPBS. After 2 washes in PBS, the stained cells on the chamber slides were mounted.

Flow Cytometry. Cells were harvested with PBS/5mM EDTA and resuspended in PBS, 10% FCS. Primary antibodies were applied at 5 µg/ml. After incubation for 30 min, the cells were washed with PBS and incubated for a further 30 min with fluorescently labelled secondary antibody. The cells were washed with PBS, then analyzed using a Becton-Dickinson FACStar Plus Flow Cytometer. For negative control samples, secondary reagent alone was routinely added, although no difference in staining was observed if nonspecific control antibodies were additionally used as primary antibodies in these controls. The data were analysed on a Hewlett Packard Consort 330 computer using Becton Dickinson FACStar Plus and LYSYS software.

Spontaneous metastasis assays. Male and female BDX rats 8-12 weeks old were given subcutaneous injections of 5×10^5 cells in PBS. Animals were regularly monitored until their tumours grew to the German legal limit or until they became moribund, at which time they were killed and an autopsy performed. In antibody therapy experiments, the tumour cells were injected into the rats together with 200 µg/ml antibody. Thereafter the animals received 200 µg/ml antibody subcutaneously at the site of the tumour cell injection twice weekly for 4 weeks.

Proliferation assay. The effect of antibodies on the proliferation of tumour cells in vitro was assessed by ^{3}H-thymidine incorporation. 1×10^5 cells were pipetted into 96 flat-bottomed microtiter plates together with antibody as appropriate in a final volume of 100µl RPMI/10% FCS and incubated for 2 days at 37°C, then ^{3}H-thymidine was added to 10 µCi/ml. After a further 12 hours incubation, the cells were harvested onto glass fiber filters with a Skatron Comi Cell Harvester and ^{3}H-thymidine uptake was assayed with a LKB Wallac liquid Scintillation Counter.

Shedding and secretion experiments

Analysis of conditioned medium. Cells were grown to confluency. The medium was removed and the cells washed once with sterile PBS which was then replaced with 5ml RPMI/10% FCS and incubated for 24 hours. The medium was harvested and pooled for each cell type. Cells and debris were centrifuged out and the medium filtered through a 0.2μm filter. The supernatant was dialysed against water. A control of 10ml RPMI/10% FCS was also dialysed. In each case the dialysate was centrifuged and lyophilised in 1ml aliquots and stored at -20°C. For examination of antigen expression, aliquots were boiled in either non-reducing or reducing sample buffer, and subjected to SDS-PAGE and western blotting.

Analysis of shedding/secretion. Cells were harvested using PBS/5mM EDTA and washed 3 times in ice cold PBS. 5×10^5 cells were resuspended in 200 μl RPMI/10% FCS containing either Nocodazole (1μg/m), 1,10-phenanthroline (5mM), TLCK (N∝-p-tosyl-L-lysine chloromethyl ketone; 200μg/ml), 3,4-dichloroisocoumarin (100μM), EDTA (5mM) or 1-2 U phospholipase C. Cells were incubated at 37 °C for various times. Incubations were terminated by centrifugation at 4000 rpm in a microfuge and the supernatant removed and lyophilised as previously described. The cell pellet and lyophilised supernatant were individually taken up in sample buffer and subjected to SDS-PAGE and western blotting. In separate experiments, cells were treated in the same manner, but the cell pellet was resuspended in ice-cold PBS and FACs analysis was performed by the previously described procedure.

Antigen purification

Affinity purification. 10AS cells were grown to confluency in 14cm petri dishes, the medium removed and replaced with 5ml RPMI 10% FCS. The plates were incubated overnight and the medium harvested. Dead cells were

centrifuged out and the medium filtered through a 0,2μm filter. The supernantant was then extensively dialysed at 4°C against PBS. The dialysed media was subsequently processed for further purification or stored at -80°C. Antibodies crosslinked to Protein G (as described above) were incubated with the dialysed media overnight at 4°C. The beads were washed 4 times with 10 volumes of PBS. Elution of bound proteins was achieved with PBS containing 1% SDS unless otherwise stated. For experiments using FPLC, elution was achieved with 50mM Tris, pH 9.0 containing 1%SDS to allow effective removal of detergent from the solution.

Eluate processing. Detergent was removed from column eluates following the instructions supplied with an Extracti-Gel® D Detergent Removing Gel purchased from PIERCE. After detergent removal the eluate was dialysed overnight in the appropiate chromatography buffer. For Mono Q and S chromatography columns (Pharmacia) the dialysis and column binding buffers were 20mM Tris, pH 7.5 and 50mM Phosphate Buffer, pH 7.0, respectively. Prior to FPLC, the eluate was incubated for at least 2 hours with protein G beads to remove any immunoglobulins that may have leached from the affinity column.

FPLC purification of the 10-1 antigen. A Pharmacia FPLC chromatography system was utilised with Mono Q and S columns. Column equilibration was performed before purification with dummy runs of the binding and eluting buffers. The eluting buffers were 20mM Tris, 1M NaCl, pH 7.5 (for Mono Q) and 50mM Phosphate Buffer, 1M NaCl, pH 7.0 (for Mono S). Once UV absorbence of zero in the column flow through was achieved after equilibration, (measured by a Pharmacia Monitor UV-M at 280nm), chromatography was performed at a flow rate of 1ml/min. For purification, the sample was injected onto the column in the respective dialysis/binding buffer at 3 min and at 5 min the eluting buffer was applied to the column in the form of a linear gradient, with its concentration increasing from 0% to 100%

over 20 minutes. 1ml fractions/minute were collected which were analysed continuously for UV absorbence. Fractions corresponding to peaks of UV absorbing material on the printed chromatogram were further analysed.

Detection of antigen. The chosen fractions were concentrated down to 80µl by Amicon centrifugal concentrators and a small portion of the products loaded onto a 10% PAGE gel in non-reducing sample buffer. Western blot was performed with anti-antigen antibodies and secondary antibody alone to distinguish the antigen from any interfering immunoglobulins or other contaminants. The remaining purified antigen was loaded onto a 10% PAGE gel 30cm in length. The proteins were transferred to PVDF membrane by western blot and the membrane was stained with coomassie blue according to the manufacture`s instructions to visualise the antigen.

vii) *N-terminal sequencing.* Samples for N-terminal sequencing were analysed at the Biotechnology Resource Laboratory, Protein Sequencing and Peptide Synthesis Facility, Medical University of South Carolina. They were analysed by Edman chemistry using a PE/Applied Procise 494 Sequenator (Edman and Begg, 1967).

Nucleic acid methods

RNA preparation and Northern blots. Tissue culture cells were harvested, washed in PBS, pelleted, then snap frozen. Polyadenylated RNA was prepared from snap frozen cells and tissues (Rahmsdorf et al., 1987). Northern blots using 1% agarose-formaldehyde gels and 5µg polyadenylated RNA were performed as previously described (Hofmann et al., 1998). Blots were probed with the rat α-casein probe at low stringency (42°C) and high stringency (65°C). After each probing, the blots were stripped in 0.1% SDS at 100°C, then exposed to film to ensure complete removal of the probe. To demonstrate equivalence of RNA loading the blots were probed with GAPDH.

Generation of rat α-casein DNA probe. 0.5μg of polyadenylated rat mammary gland RNA was reverse transcribed using Superscript Reverse Transcriptase (Life technologies) and one tenth of the reaction used for PCR. A 855 bp probe representative of all sequences coding between the N- and C-terminal ends of rat-α-casein was amplified from the cDNA by RT-PCR (30 cycles: 1 minute 94 °C; 1 minute 59 °C and 90 seconds 72 °C; a final 5 minute extension at 72 °C was performed). The primers used in this reaction for the amplification have the following sequences:

rat α-casein 62-86s: 5´-ATGAAACTTCTTATCCTCACCTGCC-3´
rat α-casein 893-917as: 5´-TCACCACACATTGGTGTTTTCAGC-3´

The positions refer to the rat-α-casein sequence as published by Hobbs and Rosen, 1982. The PCR product was gel purified and used for Northern blot analysis.

RESULTS

Subtractive immunisations using MTW9 tumour lines

A series of rat mamary tumour cell lines arising out of a common primary tumour called MTW9 have been developed by Professor Untae Kim. These differ in their metastatic proclivity (Kim and Depowski, 1975; Kim, U., 1986; Rudland et al., 1989). In order to identify metastasis-specific antigens expressed in these tumours, I performed subractive immunisations using total tumour material as the tolerogen and immunogen antigens, as not all of the MTW9 tumour lines had been established in tissue culture (during the course of the subtraction the MT-W9B cell line was established). In subtractive immunisation, B cells which proliferate in reponse to immunisation with one cell type (the tolerogen) are killed by cyclophosphamide. Subsequent immunisation with the other cell type (the immunogen) results in a humoral response directed against antigens found only on the immunogen. Immunisations of tumour material from the rat mammary tumour cell lines of MT-450 (immunogen) minus MT-W9B (tolerogen) were performed. Prebleeds and test bleeds were taken after the tolerisation and immunisation steps. Cell ELISA with tumour cells isolated from MT-W9B and MT450 tumours and these bleeds was performed to determine which mice had developed an enhanced immune reactivity to the immunogen. Five mice (numbers 3, 6, 7, 8 and 9) exhibited such an enhanced immune response (Table 6).

Fusions were made from spleens of subtractively immunised mice and hybridomas were selected by the preferential binding of antibodies they produced to the immunogen in cell ELISA assays. In total, sixty hybridomas were tested and eight selected, of which three specifically recognised tumour cells isolated from MT-W9B tumours and five from MT450 tumours. The finding that antibodies from three hybridomas reacted only with the tolerogen suggested that the subtractive immunisation was not optimal. Nevertheless,

Table 6. Differential immune reactivity of tolerogen suppressed mice after inoculation of the immunogen.

Mouse number	MT 450 ELISA OD A_{405}	MTW9B ELISA OD A_{405}	ELISA-ratio (MT450/MTW9B)
3	3.127	0.504	6.20
6	1.606	0.493	3.26
7	0.458	0.232	1.97
8	0.889	0.247	3.60
9	0.684	0.397	1.72
10	0.648	0.606	1.07

Mice were injected with tolerogen, cyclophosphamide and immunogen as described in Methods. Serum prebleeds and test bleeds were diluted 1:100, 1:200, 1:400 and 1:800 with PBS and tested in whole cell ELISA with 1AS and 10AS cells. ELISA values here represent OD_{405} values at 1:800 dilution from single experiments corrected by subtracting OD resulting from binding of preimmune sera. Ratio of the OD_{405} values for MT450/MTW9B was calculated. 10 mice were used for each subtractive immunisation and those mice that survived cyclophosphamide treatment are described here.

tissue culture supernatants from the eight hybridomas were screened for reactivity with MT-W9B and MT450 tumour cell lines by western blot, immunoprecipitation, immunofluorescence, and immunohistochemistry of sections from paraffin embedded MT-W9B and MT-450 tumours. No reactivity was observed against either tumour cell line by antibodies secreted by the hybridomas in western blots or immunoprecipitations. In immunofluorescence studies some of the eight antibodies, despite being initially chosen by cell ELISA to recognise only tumour cells isolated from one tumour, displayed non-differential reactivity by recognising the opposing tumour cell line, or both tumour cell lines, or exhibited no reactivity at all. Immunohistochemistry of sections from paraffin embedded MT-W9B and MT-450 tumours showed that antibodies specific for the immunogen recognised stromal regions of the tumour sections rather than tumour cells. When sections of tumours from other rat models of metastatic cancer were immunostained, for example the metastasising mammary carcinoma SMT-2A (Kim, 1986) and non-metastatic mammary carcinoma NM-081 (Ghosh et al.,

1983), staining was also observed in the stroma of both tumour types, demonstrating that epitopes recognised by these antibodies were not restricted to metastasising tumours.

The injection of tumour material consists of tumour cells, immune cells, endothelial cells, stromal cells and extracellular matrix components, and this complexity perhaps decreases the probability of obtaining an enhanced immune response against metastasis-specific antigens. To overcome this problem, I investigated whether tissue culture cells established at this time from the non-metastasizing MT-W9B cell (Jonathan Sleeman, unpublished results) could be used as a non-metastatic partner for MT450 cells in subtractive immunisation. Compared to the original tumour line, tumours derived from the W9B tissue cultured cell lines exhibited different morphology in paraffin sections, with marked cellularity, anaplasia, aneuploidy and giant cells. I checked the metastatic properties of this cell line to ensure that the cells were suitable non-metastatic candidates for use in a subtractive immunisation together with metastatic MT-450 cells. Syngenic spontaneous metastasis assays with various clones of the MT-W9B cell lines were performed (data not shown). From these experiments it became evident that the MT-W9B cells established in tissue culture had metastatic potential. As such, to use this cell line in a subtraction immunisation together with the metastasising MT-450 cells would be unlikely to yield antibodies against metastasis-associated antigens.

Subtractive immunisation using BSp73ASML tumour lines

To increase the chances of attaining an efficient subtraction, I decided to find another metastasis model with tissue culture cell lines having better distinguished metastasising and non-metastasizing properties. The BSp73AS pancreatic carcinoma system consists of a number of cell lines with different non-metastasising and metastasising properties (Matzku et al., 1983). In preliminary experiments, two clones from this system called 1AS and 10AS had been identified as having non-metastasising, and metastasising properties, respectively (Jonathan Sleeman, unpublished observation). I performed syngenic spontaneous metastasis assays with these cells and found that the 1AS cells did not metastasise, while the 10AS cells formed metastases in the lymph nodes and/or lungs of BDX rats (Table 7). These results demonstrated that these two related cell lines have clear and distinguishable metastasising properties, suggesting that they would be suitable candidates for a subtractive immunisation.

Table 7. Metastatic properties of 10AS and 1AS cell lines.

Cell line	Growth in Rat	Lymph node	Lung
10AS	8/8	7/8	1/8
1AS	8/8	0/8	0/8

BDX rats (for syngenic spontaneous metastasis assays) were injected subcutaneously with 5 x 10^5 tumour cells in PBS. Animals were regularly monitored until their tumours grew to the German legal limit or they became moribund, at which time they were killed and an autopsy performed.

Mice were subtractively immunised with non-metastatic 1AS cells as the tolerogen followed by the metastatic 10AS cells as the immunogen. Prebleeds and test bleeds were taken after the tolerisation and immunisation steps. To determine which mice had developed differential immune reactivity towards the immunogen, cell ELISA was performed with the prebleeds and test bleeds using 1AS and 10AS cells as targets. Mouse 9 had the highest 10AS/1AS ELISA ratio of 2.36, indicating that cyclophosphamide had suppressed the

immune response to non-metastasising 1AS cells and permitted an enhanced differential response to metastasising 10AS cells (Table 8). Mouse 5 had a slightly elevated immune response and was also chosen to increase the number of total splenocytes in the fusion reaction and thus increase the efficiency of generating hybridomas.

Hybridomas were produced by fusing immune splenocytes from mouse 5 and 9 with SP2/0 mouse myeloma cells, and the supernatants were tested by cell ELISA to see if they contained antibodies which bound to 1AS and 10AS cells. It was observed that none of the hybridomas recognised 1AS cells (tolerogen) alone, demonstrating the effectiveness of the subtractive immunisation. In total, 39 hybridomas were tested and four of them 3A7, 4F3, 3E12 and 3F12 secreted antibodies which recognised only the 10AS metastatic cells (Fig. 1). These hybridomas were used for further analyses.

Table 8. Differential immune reactivity of tolerogen suppressed mice after inoculation of immunogen cells.

Mouse number	10AS ELISA OD A_{405}	1AS ELISA OD A_{405}	ELISA ratio (10AS/1AS)
2	2.947	2.935	1.00
3	2.493	2.778	0.90
4	2.664	3.017	0.88
5	2.951	2.872	1.03
8	2.733	3.332	0.82
9	2.298	0.975	2.36

Mice were injected with tolerogen, cyclophosphamide and immunogen as described in methods. Serum prebleeds and test bleeds were diluted 1:100, 1:200, 1:400 and 1:800 with PBS and tested in whole cell ELISA with 1AS and 10AS cells. ELISA values here represent OD_{405} values at 1:800 dilution from single experiments corrected by subtracting OD resulting from binding of preimmune sera. Ratio of the OD_{405} values for 10AS/1AS was calculated. 10 mice were used for each subtractive immunisation and those mice that survived cyclophosphamide treatment are described here.

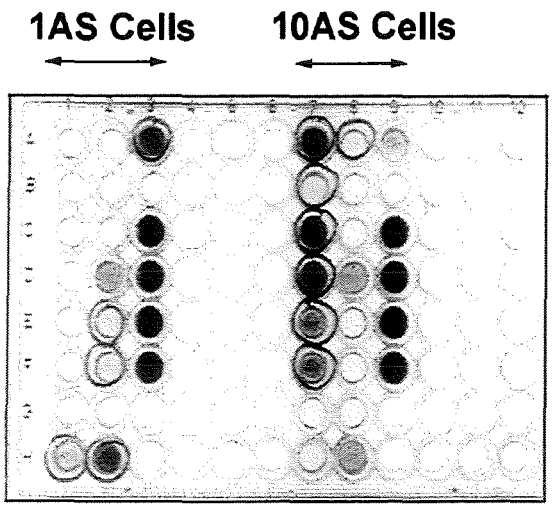


Figure 1. ELISA plate of final hybridoma supernatants from subtractive immunisation. Hybridoma supernatants in wells A7, C7, D7 and E7 represent hybridomas 3A7, 3E12, 3F12 and 4F3 respectively, and exclusively recognised 10AS cells in comparison to 1AS cells in wells A1, C1, D1 and E1.

Characterisation of the 10AS-specific antibodies

The monoclonal antibodies 3A7, 4F3, 3E12 and 3F12 bind to 10AS cells, but not 1AS cells, suggesting that the antigen they bind to may be specific for metastasising cells. To characterise the antigens recognised by the antibodies present in the hybridoma supernatants, I checked the reactivity of these antibodies in various assays. 10AS and 1AS whole cell lysates were electrophoresed in reducing SDS-PAGE gels and probed by western blot with supernatants from the four hybridomas. The antibodies produced by the hybridomas all recognised a group of antigens migrating at 65-80 kDa which are present only in 10AS cells (Fig. 2). FACS analysis showed that the Mabs produced by these hybridomas bound strongly to antigens present on 10AS cells, whereas only slight binding was observed with 1AS cells (Fig. 3). Cell staining studies of 10AS and 1AS cells revealed that each hybridoma secreted antibodies which reacted strongly and exclusively with the metastatic 10AS cells (Fig. 4). On the basis of their differential reactivity for antigens present on 10AS cells these hybridomas were chosen for further studies. They were put into mass culture and the secreted antibodies purified by affinity binding to protein G. The purified antibodies 3A7 and 3E12 were immunotyped as IgG_1 λ, while 4F3 and 3F12 were found to have IgG_3 κ subclass (Fig. 5).

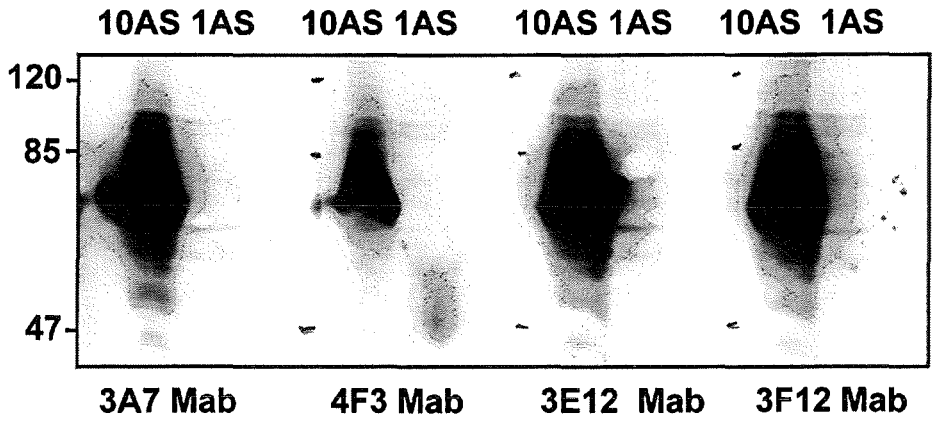


Figure 2. Western blot analysis of immunodeterminants present on 10AS and 1AS cells. Lysates from 1×10^5 cells were loaded under reducing conditions into each lane of an 8% PAGE SDS gel. The blots were probed with supernatants from hybridomas 3A7, 4F3, 3E12 and 3F12, and showed differential antigen expression by 10AS cells.

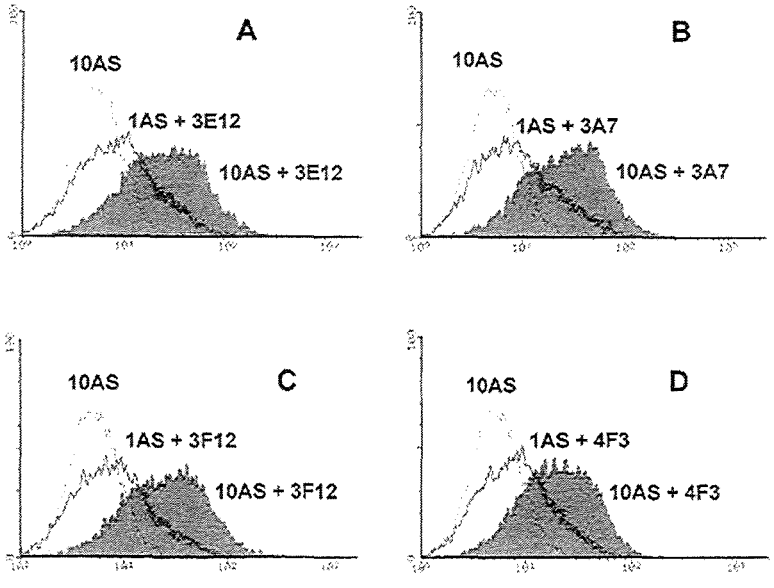


Figure 3. FACS analysis of differential surface expression of immunodeterminants present on 10AS cells recognised by supernatants from 3A7, 4F3, 3E12 and 3F12 hybridomas. Plots of fluorescence intensity (*absicssa*, log scale) against cell number (*ordinate* , linear scale) are shown.

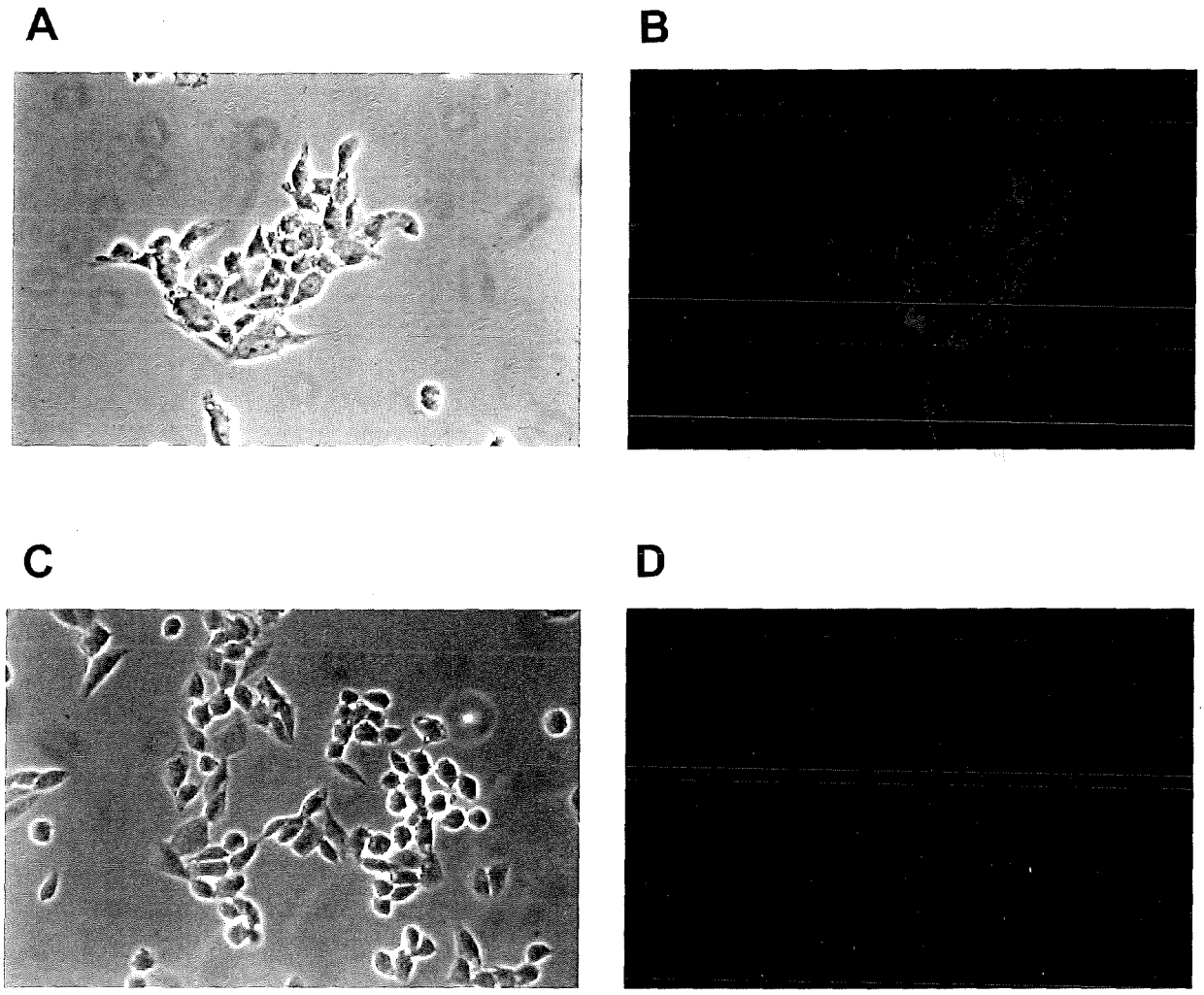


Figure 4. Phase contrast (A and C) and immunofluorescence (B and D) images of 10AS (A and B) and 1AS cells (C and D) stained with the 3A7 antibody. Cells were cultured in 2% FCS RPMI and treated with PFA and 1% NP40 prior to staining. Only 10AS cell stain with the 3A7 antibody

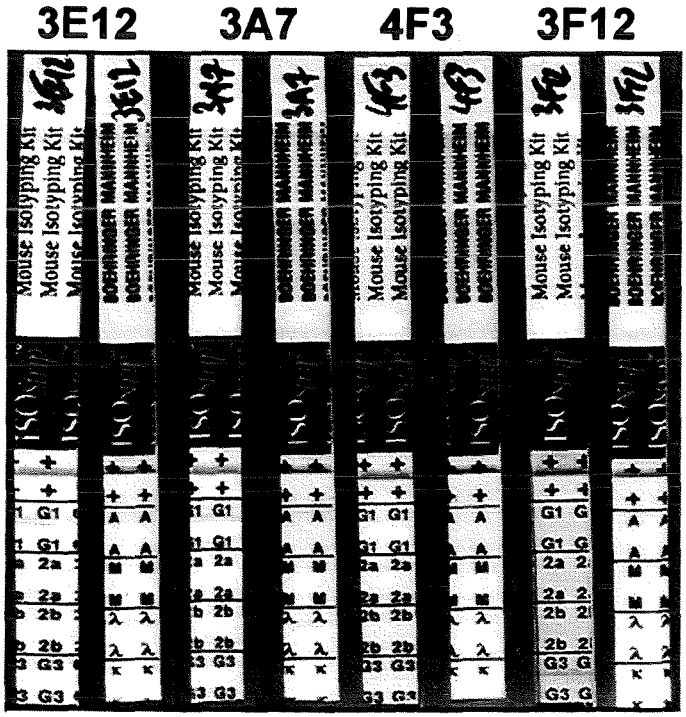


Figure 5. Isotypes of monoclonal antibodies 3E12, 3A7, 4F3 and 3F12. An ISO*Strip*™ monoclonal anti-body isotype kit from Boehringer Mannheim was used according to the manufacturer's instructions. Isotypes tested were IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgM and kappa and lamba light chains. 3E12 and 3A7 were isotyped as IgG_1 λ and 4F3 and 3F12 as IgG_3 κ subclasses.

The monoclonal antibodies 3A7, 4F3, 3E12 and 3F12 bind to a common antigen

In initial experiments to test the reactivity of the hybridoma supernatants on western blots, I detected a group of antigens with varied expression over a 65-80 KDa range (Fig. 1). I repeated these experiments and probed the western blots with the purified antibodies. I observed that there was little difference in the recognition by each antibody of antigens expressed by 10AS cells migrating at 65-70 kDa, implying that the four antibodies bind the same antigen (Fig. 6A, B, C and D). To confirm this, I peformed immunoprecipitations with each of the Mabs, loaded the products on a SDS PAGE gel, blotted and probed for antigen expression with each monoclonal antibody. I observed that each Mab recognised the same migrating protein, indicating that they bind to the same antigen (data not shown). It must also be said that each of the four antibodies had different affinities in immunoprecipitations, suggesting that they recognise different epitopes and are not alike. Hereafter, these antibodies are referred to as the 10-1 antibodies and the antigen to which they bind is called the 10-1 antigen.

In initial experiments with hybridoma supernatants no immunodeterminants were recognised in 1AS cell lysates. With purified 10-1 antibodies I detected minor components not seen with hybridoma supernatants (Fig. 6E) consistent with the slight staining of 1AS cells observed in FACs staining. These minor protein components migrate as a doublet of 65-75 kDa and represent only a small component of the total 10-1 antigen in 10AS cells. This doublet was recognised by all four 10-1 antibodies (data not shown).

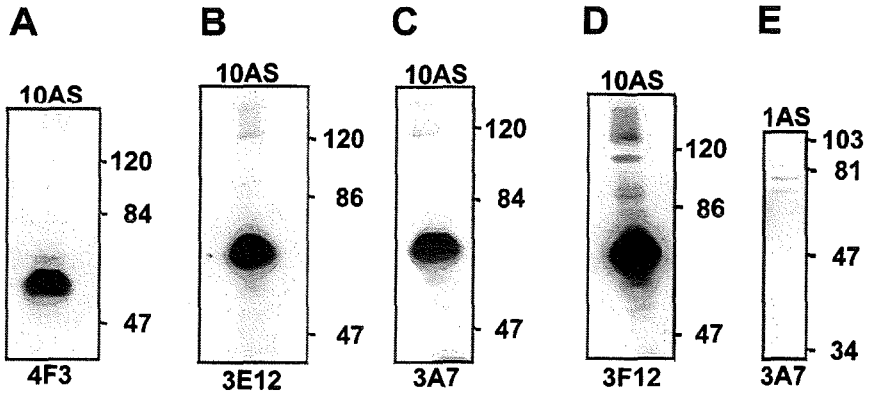


Figure 6A to D. 10-1 antibodies recognise the same antigen. 10AS lysates were separated on 6% SDS PAGE gels, blotted and probed with the indicated antibody. **E.** Recognition of a 65-75 kDa doublet by 10-1 antibodies in 1AS cell lysates. Lysates were separated on a 10% SDS PAGE gel, blotted and probed with antibody 3A7.

Localisation of the 10-1 antigen on 10AS cells

Initial experiments showed that the 10-1 antigen was expressed by metastatic 10AS cells. To get some clues as to where this antigen functions in cells, confocal microscopy was perfomed (Open lab Scientific Imaging System) to visualise where the 10-1 antigen is localised on 10AS cells. Examination of antibody staining with this technique suggested two interesting features, namely the staining localised to what appeared to be filopodia and round structures on the cell membrane (Fig. 7). Filopodia are a common feature of animal cells, especially when they are moving or changing shape. For example they are utilised by metastatic cells to invade other tissue sites and travel throughout the ECM. The round structures are reminiscent of secretory vesicles, suggesting they may be sites of 10-1 release at cell membrane and thus that the 10-1 antigen may be secreted. Metastatic tumour cells release a plethora of molecules that facilitate, for example, the breakdown of ECM components. These observations preliminarily suggested but did not prove that the 10-1 antigen may be involved in facilitating cell migration and/or invasion.

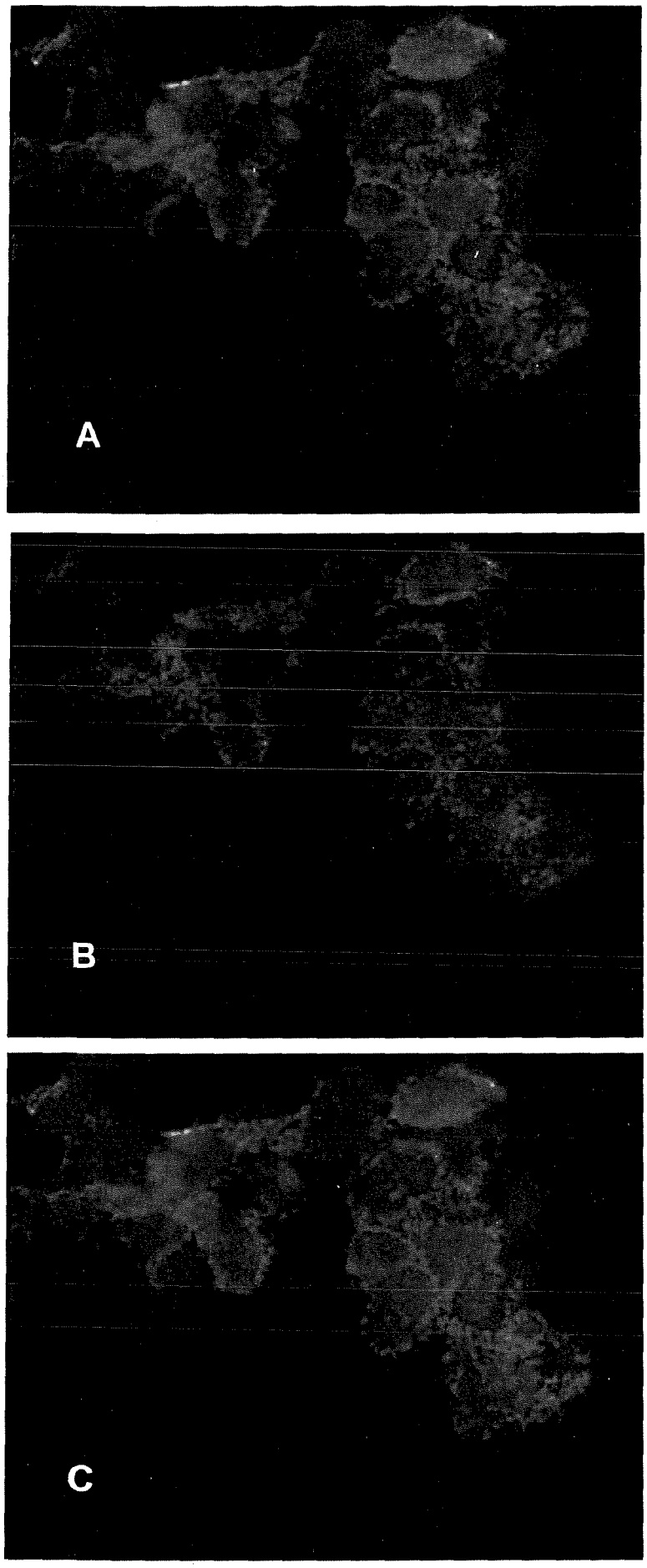


Figure 7. Confocal immunofluorescence images of 10AS cells stained with Mab 3A7. Cells were cultured in 2% FCS RPMI and treated with PFA and 1% NP40 prior to staining. **A.** Basolateral view. Staining is localised to filopodia **B.** Medial view. Staining is localised to round structures which is suggestive of secretory vesicles. **C.** Apical view. Localised staining to filopodia

Biochemical characterisation of the 10-1 antigen

To begin to elucidate a function for the 10-1 antigen on 10AS cells I began to further biochemically characterise the antigen. In different experiments with apparently similarly prepared 10AS cell lysates I sometimes observed different migration patterns of the 10-1 antigen. For example, Figures 8A, 8B and 8C show the migration of the 10-1 antigen from three separate cell lysate preparations. Each western blot was probed with the antibody 4F3, and protein bands were observed migrating at 60-70 kDa, 50-58 kDa and 40-80 kDa, respectively. The same differences in antigen migration were visualised by each of the 10-1 antibodies (data not shown). To determine exactly how many 10-1 antigen isoforms are present in 10AS cells, cell lysates were separated on a 5-20% SDS PAGE gel and the western blot probed with the 3A7 antibody. It was observed that a group of proteins which migrated over a wide range of 28-80 kDa were recognised by the antibody (Fig. 9). One of several explanations for these multiple forms could be that the antigen is subjected to some form of proteolytic processing. Events of this nature are often associated with the formation of secretory vesicles (Neurath, 1991). These observations combined with evidence from cell staining studies suggesting the 10-1 antigen may be present in secretory vesicles on the surface

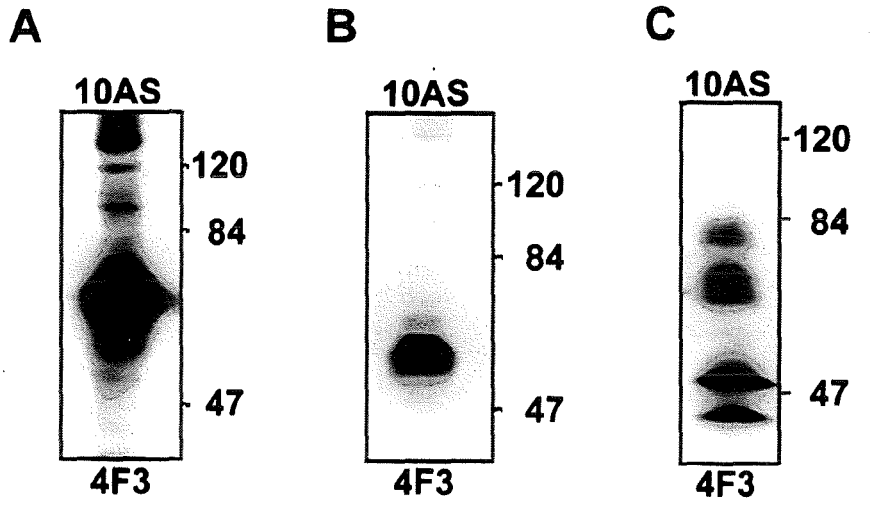


Figure 8A to C. 10-1 Antigen is expressed in different forms. In a series of independent experiments 10AS cell lysates were separated on 6% SDS PAGE gels, blotted and probed with antibody 4F3.

of 10AS cells, adds weight to the hypothesis that an active secretory system could be responsible for transporting the 10-1 antigen to and releasing it from the cell surface. The aspect of 10-1 antigen secretion will be considered in later sections.

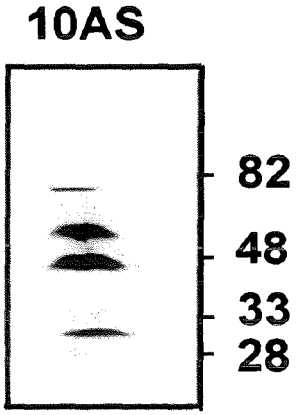


Figure 9. Multiplicity of 10-1 antigen forms. 10AS lysate was separated on a 5-20% SDS PAGE gel, blotted and probed with antibody 3A7.

To determine whether disulphide-bridges or glycosylation contribute to the different protein isoforms of 10-1 antigen, 10AS cells were treated with dithiothreitol (DTT), tunicamycin, O-glycosidase and neuraminidase. DTT breaks the disulphide bonds that hold the protein subunits of multi-subunit molecules together. It had no effect on the migration of the 10-1 antigen from 10AS cell lysates and supernatants on SDS PAGE gels, indicating that the antigen is composed of single polypeptide chains (Figs. 10B and 10C). Tunicamycin inhibits N-glycosylation by blocking the first step in the biosynthesis of the lipid-linked oligosaccharide precusor (the synthesis of dolichol pyrophosphate N-acetylglucosamine). Treatment of 10AS cells with tunicamycin did not alter the migration pattern of the 10-1 antigen, demonstrating that the antigen is not modified by N-linked sugars (Fig. 10). Furthermore, experiments with O-glycosidase and neuraminidase did not provide any evidence of other sugar modifications on the 10-1 antigen (data not shown). These data indicate that the multiple forms of the 10-1 antigen do not represent disulphide-linked multimers or differentially glycosylated products.

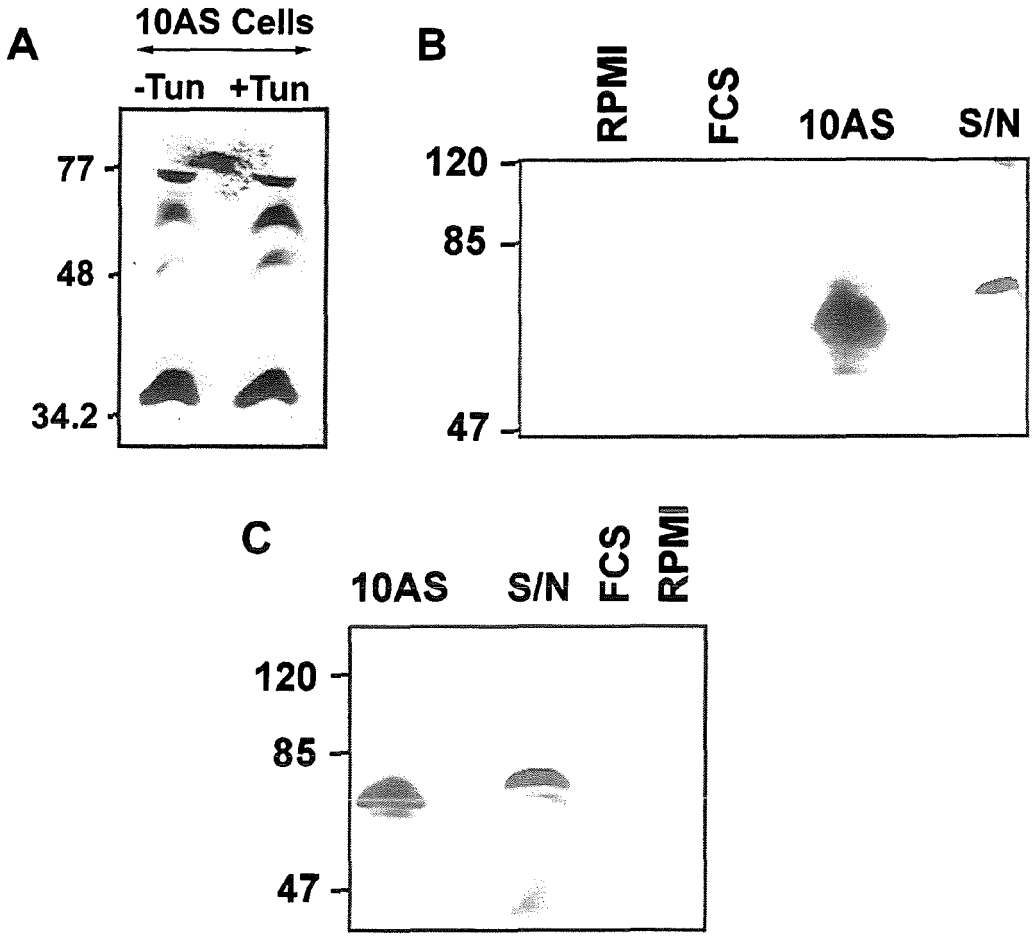


Figure 10A. The antigen is not modified by N-linked sugars. Lysates of 10AS cells cultivated in the presence (+Tun) or absence (-Tun) of tunicamycin, separated on a 10% SDS PAGE gel, blotted, and probed with Mab 3A7. **B and C.** The 10-1 antigen is composed of a single polypeptide chain. Supernatants (S/N) from 10AS cells (10AS) grown in 10% FCS/RPMI, and controls of RPMI and 10% FCS/RPMI, were dialysed against water and lyophilised. 5µl equivalents were loaded into each lane of a reducing (Fig. 10B) or non-reducing (Fig. 10C) 8% SDS PAGE gel, electrophoresed, blotted and probed with Mab 3A7.

The 10-1 antigen is down-regulated *in vivo*

The 10-1 antibodies were produced on the basis of their ability to bind to a metastasising but not to a non-metastasising pancreatic carcinoma cell line. In order to determine whether differential expression is exhibted *in vivo*, I performed immunohistochemistry using 10AS primary tumours, 10AS lymph nodes containing metastatic 10AS cells, and 1AS primary tumours.

I initially attempted to stain paraffin sections with the 10-1 antibodies. The best observed staining was a possible weak signal in the tumour stroma

regions but this was associated with an elevated background staining. To attain cleaner staining, I altered the standard procedure by using more effective blocking agents, such as 1% immunoglobulin free BSA and CAS BLOCK™ (ZYMED). I also reduced endogenous peroxidase activity by pretreating the sections with 0.1% phenylhydrazine or stronger concentrations of hydrogen peroxide, and blocked endogenous biotin activity (Biotin Blocking System, DAKO). A high background with these method alterations persisted. I therefore changed the detection system to alkaline phosphatase (DAKO APAAP KIT™) and encountered similar background problems. I thus again changed the detection system to β-galactosidase and encountered very weak positive control staining by the Mab 5G8 for CD44 in both 10AS and 1AS sections (which was strong in the other methods), but obtained no staining by the 10-1 antibodies. The poor control staining made this detection system inappropriate for further histochemical investigation. These methodology problems may suggest that the 10-1 antibodies do not work on paraffin sections. Alternatively, 10-1 antigen expresion *in vitro* may be a tissue culture phenomenon and *in vivo* 10-1 antigen expression is perhaps down regulated. The 10-1 antigen may also be actively released from the cells *in vivo* as suggested by the confocal studies and therefore not be detectable as it is rapidly cleared from the tumours. I investigated these issues further.

To determine whether the 10-1 antibodies do not work on paraffin sections, I performed histochemistry using frozen sections. Similar problems of elevated background in peroxidase and alkaline phosphatase procedures and poor control staining in the β-galactosidase method were encounted. I tried to overcome these inconsistencies by using the above described method variations, however this proved to be unsuccessful.

Finding that histochemistry optimization was difficult and knowing that the 10-1 antibodies worked well on western blots, I checked for 10-1 antigen expression in western blots using lysates of 10AS tumour material, metastasising lymph nodes, and *in vivo* passaged tumour cells in tissue culture from the primary tumour and lymph nodes with metastases (Fig. 11). It was

observed *in vivo* that the 10-1 antigen was down-regulated in tumours and metastasising lymph nodes. The tumours and lymph nodes were removed, returned to tissue culture and checked for 10-1 antigen expression. I found that in comparison to long-term tissue culture of 10AS cells, 10-1 antigen expression in freshly repassaged 10AS tumour cells was down regulated. It must also be said that the 10-1 antigen present in the 10AS tumour, 10AS lymph node and passaged tumour cells, migrated differently as a single band of 60-65 kDa compared to 10AS cell lysate, suggesting that there is a different form of 10-1 antigen *in vivo*. These results argue that 10-1 antigen expression and is down regulated *in vivo* as compared to *in vitro* expression. This could at least partly explain for the poor 10-1 antibody staining on paraffin and frozen sections.

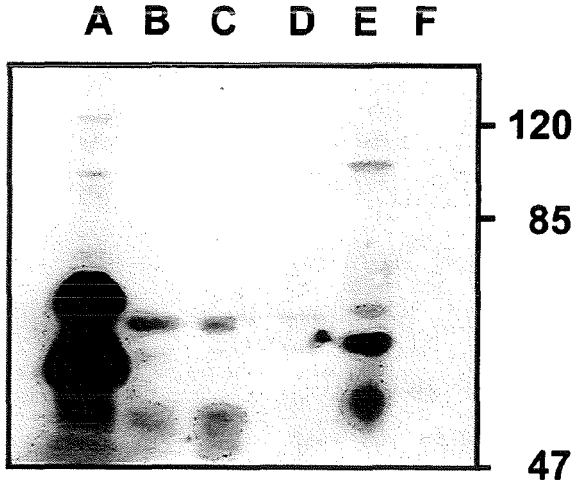


Figure 11. Antigen expression is down regulated *in vivo*. Western blot of 10-1 antigen detection in (A) 1×10^5 10AS cells, (B) 200µg 10AS tumour tissue, (C) 200µg 10AS lymph node tissue, (D) 200µg 1AS tumour tissue, (E) 1×10^5 10AS passaged tumour cells, and (F) 1×10^5 10AS passaged lymph node cells (equal loading was checked by staining the bottom portion of the gel with Coomassie (data not shown). Electrophoresis was performed on a 6% SDS PAGE gel under reducing conditions and the blot probed with Mab 3A7.

10-1 antigen expression is regulated by growth conditions

Cells in tumours experience different growth conditions as compared to those grown in tissue culture. Tissue culture medium contains foetal calf serum (FCS), so there is a plentiful supply of growth factors and nutrients. In a tumour, the supply of nutrients is limited by a restricted vascular system. To investigate whether this would explain why the 10-1 antigen is down-regulated *in vivo* I replicated restrictive growth conditions *in vitro* by culturing 10AS cells in medium containing lower FCS concentrations. Cell lysates were

prepared from these cells, subjected to SDS-PAGE and western blot, and probed with the Mab 3A7. Surprisingly in low serum (0-2% FCS/RPMI) the cells exhibited high 10-1 antigen expression and in high serum (10% FCS/RPMI) comparatively little 10-1 antigen expression was seen (Fig. 12). Therefore down-regulation of the 10-1 antigen in the tumour is not due to poor nutrient conditions. This result, combined with the weak *in vivo* expression demonstrated by western blot, could suggest that growth conditions regulate expression of the 10-1 antigen. Alternatively growth conditions could determine whether the 10-1 antigen remains in the cell or is shed or secreted.

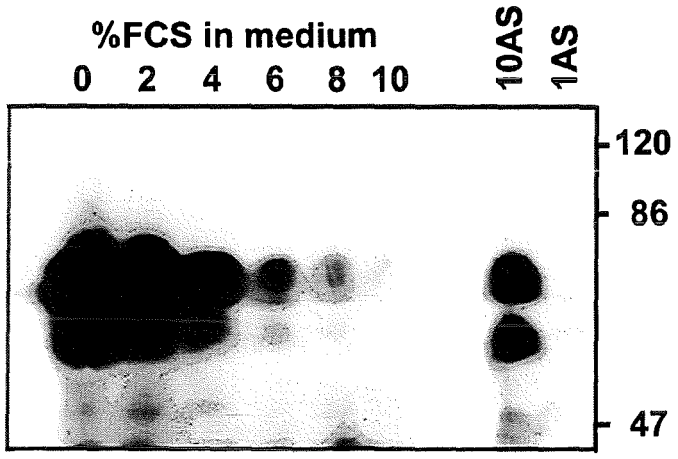


Figure 12. Antigen expression *in vitro* is regulated by growth conditions. Western blot of 10AS cells grown to 80-90% confluency for 48 hours in 0-10% FCS/RPMI. Lysates from 1×10^5 cells were loaded in each lane of a 6% gel, electrophoresed, blotted and probed with Mab 3A7.

The 10-1 Antigen is secreted

Protein shedding can occur by enzymatic cleavage of the protein backbone or glycosylphosphatidylinositol (GPI) linkages. I investigated whether or not release of the 10-1 antigen from 10AS cells was due to these processes. I found that chemical inhibition of enzymatic cleavage or the addition of phospholipase C to break GPI linkages had no effect on 10-1 antigen retention or release (data not shown), suggesting that the 10-1 antigen is not shed from 10AS cells.

Secretion of proteins can occur immediately after synthesis and packaging of proteins into secretory vesicles (constitutive exocytosis) or,

following variable periods of storage in secretory vesicles or granules, in response to cell activation (regulated exocytosis; Burgess and Kelly, 1987). Several pieces of experimental data presented so far, such as the confocal study, the observation of multiple forms of the 10-1 antigen and the effect of FCS concentration suggested that the 10-1 antigen may be secreted. To investigate this point, I performed the same experiment as shown in Figure 12 in which cells were grown in different FCS concentrations and in addition collected the conditioned media. After 48 hours the cells in 0-2% FCS/RPMI expressed a high level of the 10-1 antigen, while cells in 10% FCS/RPMI reduced expression was observed (data not shown), confirming the previous result. The cell supernatant in comparison, showed low levels of 10-1 antigen expression in 0-2% FCS/RPMI, and high levels of 10-1 antigen expression in

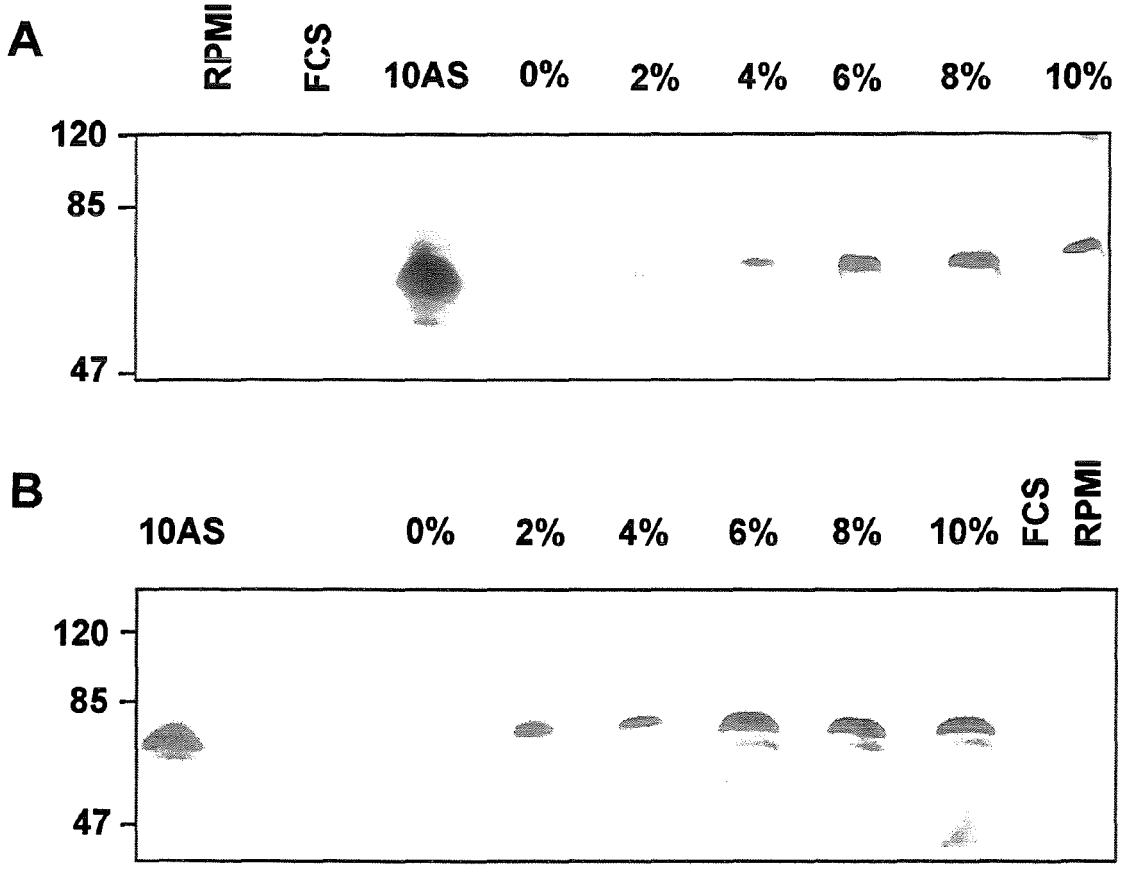


Figure 13A and B. 10-1 antigen secretion is regulated by growth conditions. Supernatants from 10AS cells grown in 0-10% FCS/RPMI, and controls of RPMI and 10% FCS/RPMI, were dialysed against water and lyophilised. 5µl equivalents were loaded into each lane of a reducing (Fig. 12A) or non-reducing (Fig. 12B) SDS PAGE 8% gel, electrophoresed, blotted and probed with Mab 3A7.

10% FCS/RPMI (Fig. 13). These data suggest that in restrictive growth conditions the 10-1 antigen remains surface bound or intracellular and in more optimal conditions the 10-1 antigen is secreted by some active process.

There are also no differences in the molecular weight of the 10-1 antigen in cell lysates and media supernatant, adding weight to the argument that an active secretory process for 10-1 antigen release is in play (Fig. 13). If enzymatic cleavage was responsible for releasing the 10-1 antigen one would expect a smaller form to be present in the media supernatant.

The 10-1 antigen is located in the cytoplasm and near the cell membrane.

To confirm that the 10-1 antigen is indeed present in the cell membrane and cytoplasm, differential detergent experiments were performed. I used the detergents Tween 20, NP40 and SDS which have particular solubilizing properties for proteins present in cell membranes (Neugebauer, 1994). Tween 20, a non-ionic detergent, is the mildest of these detergents and its action is to remove phospholipids from the cell membrane and solubilise membrane bound proteins. NP40 is a non-ionic triton detergent which solubilises protein present in the cell membrane and cytoplasm. SDS, a strong anionic detergent, readily solubilises nearly all proteins, and breaks down the nuclear membrane. Cell lysates were made with each detergent and incubated on ice for various times to observe if greater solubilisation or degradation of the 10-1 antigen occurred over time. Lysates were subjected to SDS PAGE and western blot and probed with Mab 3A7 (Fig 14A and B). After Tween 20 solubilisation, lysates exhibited two 10-1 antigen bands migrating at 45 and 60 kDa. NP40 treatment solubilised much more antigen than Tween 20 indicating that the majority of the 10-1 antigen (some 90%), is present in the cytoplasm, whereas only about 10% of the 10-1 antigen is present at or near the cell membrane, as indicated by Tween 20 treatment. SDS detergent treatment visualised total 10-1 cellular

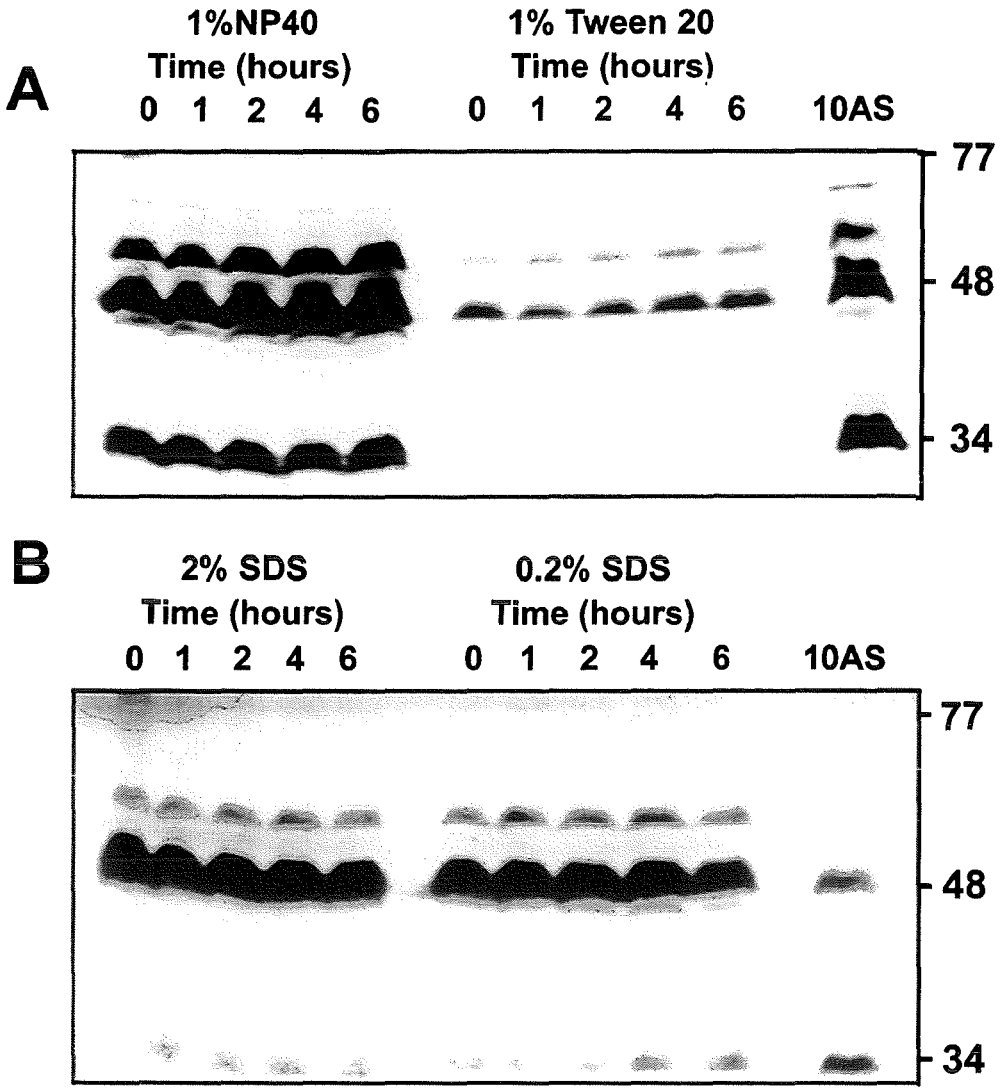


Figure 14A and B. Membrane association of 10-1 antigen. Cells were incubated with the respective detergent on ice for the indicated time. The resulting lysates were loaded onto 10% SDS PAGE gels, blotted and probed with antibody 3A7. Control of 10AS cells lysed in non-reducing sample buffer is present in the far right lane of each figure.

antigen whose molecular weight ranged from 30-60 kDa. There are no differences between the amount of 10-1 antigen solubilised by NP40 or SDS, which indicates that the 10-1 antigen is present in the cytoplasm and cell membrane and not in the cell nucleus.

To visualise any changes in localisation of the 10-1 antigen in reponse to growth conditions, cell staining experiments were performed. Cells were grown in either 2% FCS/RPMI or 10% FCS/RPMI, and subjected to fixation alone to observe membrane-associated 10-1 antigen, or to fixation followed by

permeabilisation with 1% NP40 to visualise internalised 10-1 antigen (Fig. 15). In experiments with fixation alone, cells grown in reduced serum had more 10-1 antigen localised to the cell membrane and intracellular compartments than cells grown in non-restrictive growth conditions, demonstrating that factors within the FCS have an effect on 10-1 antigen localisation and secretion.

These results support the earlier microscopy data that the 10-1 antigen is localised to vesicle-like structures and is consistent with the biochemical evidence of its intracellular localisation. Furthermore, it also suggests that changes in growth conditions can instigate the release of the 10-1 antigen by some mechanism. As such, if tumours predominantly secrete the 10-1 antigen, little 10-1 antigen may be present in histological sections to be stained by the 10-1 antibodies.

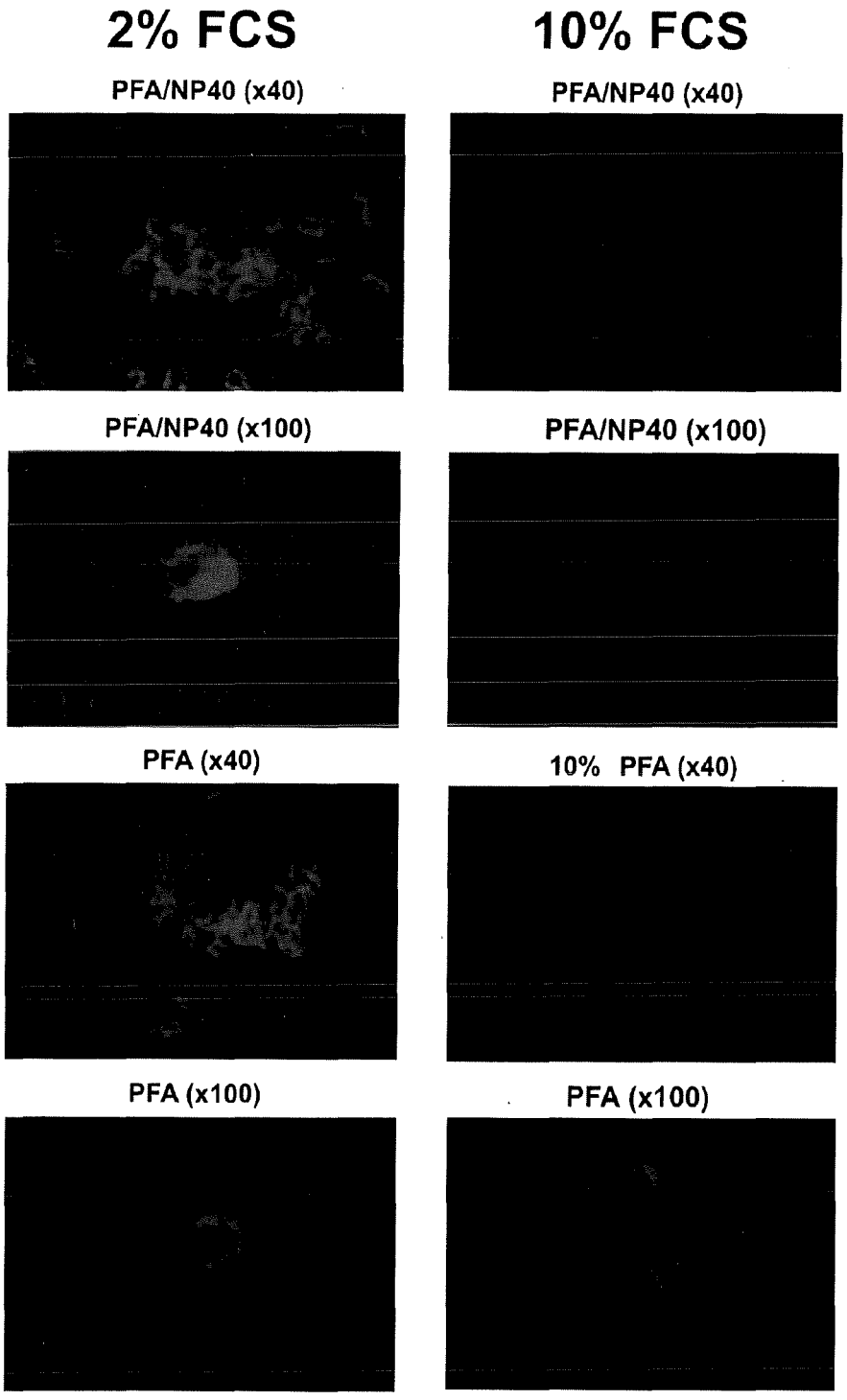


Figure 15. Differences in antigen localisation in response to growth conditions. Cells were grown in 2% or 10% FCS RPMI and treated with PFA alone, or PFA plus 1% NP40 and stained with antibody 3A7. Cells grown in 2% FCS RPMI have more antigen localised on their cell membranes and in their cytoplasm as compared to those grown in 10% FCS RPMI.

10-1 antigen expression in other members of the BSp73AS family

The BSp73 pancreatic carcinoma cell system consists of clones with different metastatic potentials. 0AS, 3AS, 7AS, 10AS and ASML have all been observed to metastasize, while 1AS is non-metastatic (Sleeman, unpublished observations). I checked this cell family for a correlation between 10-1 antigen expression and release, and their metastatic potential. I found that the 10-1 antibodies all recognised the minor doublet of proteins migrating at 65-75 kDa in lysates from 0AS, 3AS and 7AS cell lines, which had previously been observed in 1AS cells (Fig. 16A). However, the major 10-1 antigen forms expressed in 10AS cells were not observed in lysates from 0AS, 3AS or 7AS cells. The binding of the 10-1 antibodies to the 65-75 kDa doublet was not due to cross-reactivity from the secondary-HRP-labeled antibody with proteins in 1AS cell lysates (Fig. 16B).

These results demonstrate that the 10-1 antigen is upregulated and present in a variety of different forms in 10AS cells (Fig. 8), whereas in the other BSp73AS family members only a minor doublet is weakly expressed. Furthermore, upregulated expression of the 10-1 antigen is not obligatory for the metastatic properties of BSp73 family members.

The 10-1 antigen is expressed by other rat carcinomas

The 10-1 antigen was identified using antibodies made by subtractive immunisation to identify differences between metastasising (10AS) and non-metastasising (1AS) cells. To determine to what extent expression of the 10-1 antigen is correlated with metastasis, rat tumour cell lines with different metastatic potentials were tested for antigen expression by western blot. I cultivated these cell lines in 2% FCS/RPMI and 10% FCS/RPMI to see if there was a similar regulation of antigen expression by nutrient conditions *in vivo*. It was observed that there was no strict correlation of antigen expression with metastatic potential. For example, non-metastasising CREF and NM081 cells

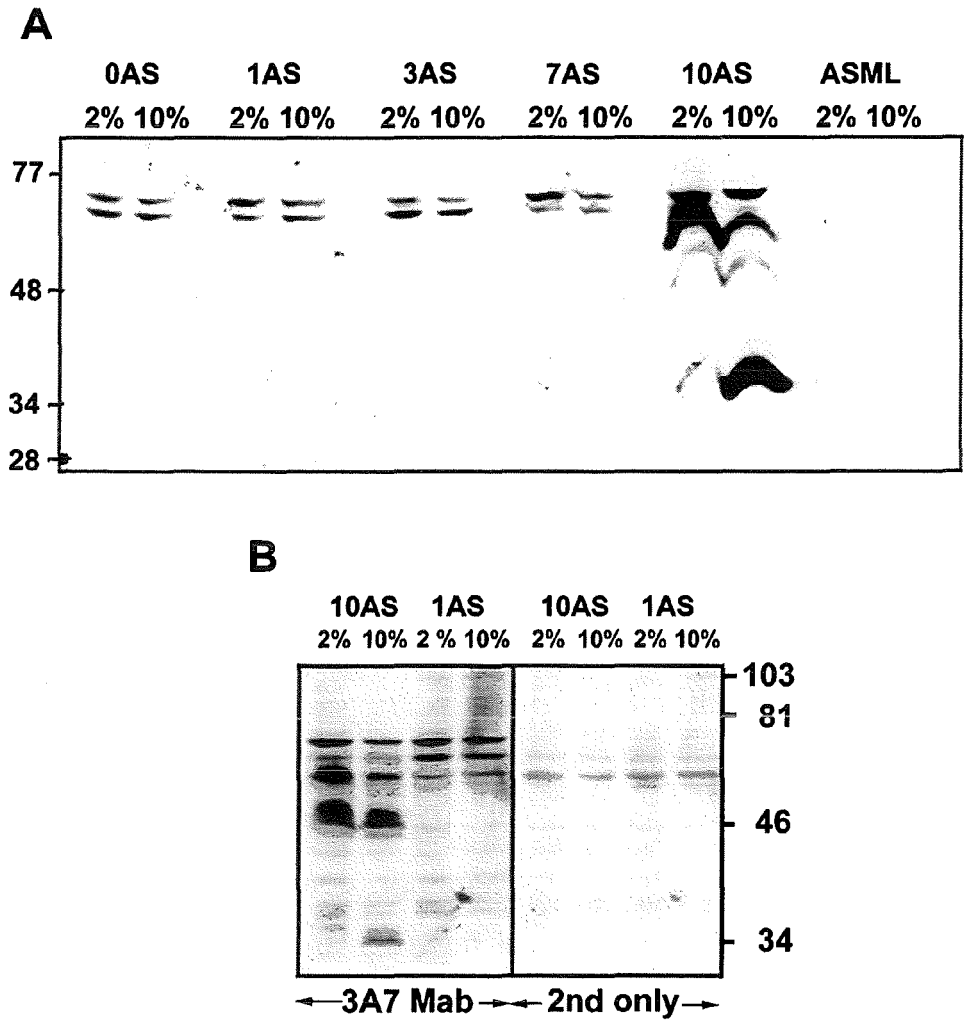


Figure 16A. Western blots of antigen expression from BSp73ASML pancreatic carcinoma cell clones. The indicated cell line was cultivated in 2% or 10% FCS/RPMI, cells lysed, 1×10^5 cell equivalents loaded on a 10% SDS PAGE gel, blotted and probed with Mab 3A7. **B.** Checking cross-reactivity of secondary-HRP-labeled antibody on 10AS and 1AS cells. 10AS and 1AS cells were cultivated in 2% or 10% FCS/RPMI, cells lysed, 1×10^5 cell equivalents loaded on a 10% SDS PAGE gel, blotted and probed with Mab 3A7 and the secondary antibody alone.

expressed 10-1 antigen, while metastasising MTLN3 and MTLy cells did not, suggesting that 10-1 antigen is not obligatory for tumour growth and metastasis in rat pancreatic, mammary or prostate carcinoma cells (Table 9; Figs. 17A, B and C).

The 10-1 antigen migrated differently in all cell lines examined. The 10-1 antigen in 10AS cells is expressed as a number of bands migrating between 34-70 kDa, whereas in the cell lines tested a single broad band or duplet as in MTC, MatLyLu and AT3.1 cells of 60-65 kDa is observed. Some cells expressing the 10-1 antigen, such as MTC, AT-1, AT2-1, and MatLu, exhibited differences in antigen levels in different growth conditions. For these cell lines more antigen is expressed at 10% FCS/RPMI and less at 2% FCS/RPMI, suggesting that growth conditions have similar regulatory effects on 10-1 antigen expression as which was earlier observed in 10AS cells (Fig. 12). Antigen secretion from these cells was not tested.

Table 9. Rat tumour cell lines that express the 10-1 antigen.

Cell line	Metastatic grading	10-1 Antigen expression
10AS	+++	Yes
1AS	-	Yes
0AS@	+++	Yes
3AS@	+++	Yes
7AS@	+++	Yes
ASML	+++	No
AT-1	++	Yes
AT-2.1	++	Yes
AT-3.1	+++	Yes
AT-6.1	+++	Yes
CREF	-	Yes
CREF T24	+++	Yes
MT450	+++	Yes
MTPa	-	No
MTC	+	Yes
MTLN2	++	Yes
MTLN3	+++	No
MTLy	+++	No
MTLu	+++	Yes
Mat LyLu	+++	Yes
BDX#2	-	No
NM081	-	Yes

Expression of 10-1 antigen on various rat tumour cell lines grown for 48 hours in 10% FCS/RPMI. Metastasis is graded according to spontaneous metastasis assays as follows: - is no metastases; + is <25% of the animals with metastases; ++ is 25-75% of the animals with metastases; +++ is >75% of the animals with metastases (adapted from Sleeman et al., 1996; @ Sleeman unpublished observations).

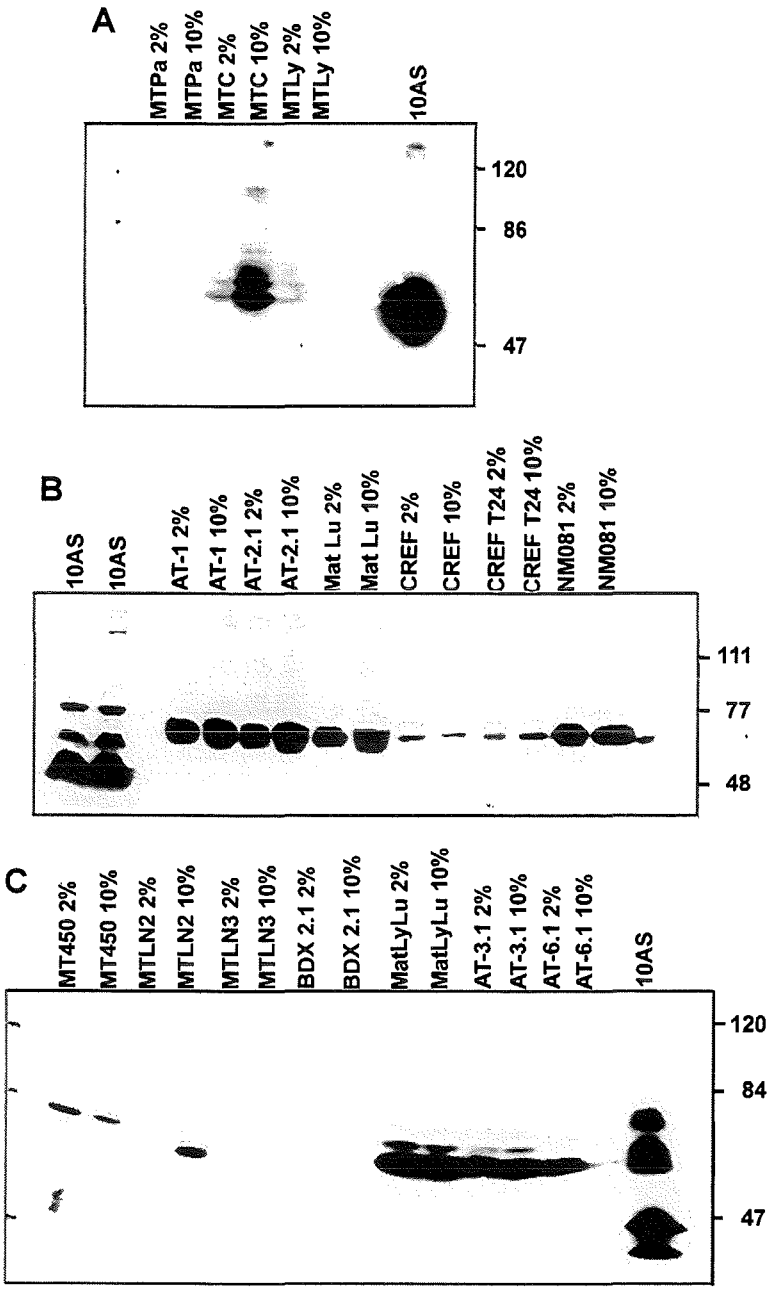


Figure 17. Western blots of antigen expression in rat carcinoma cell lines grown in 2% or 10% FCS/RPMI. **A.** Cell lysates 1×10^5 cells, were loaded on a 8% SDS PAGE gel, blotted and probed with Mab 4F3. Antigen presence in MTLy 2% is due to spillage from MTC 10%. **B.** Cell lysates 1×10^5 cells, loaded on a 10% SDS PAGE gel, blotted and probed with 3A7. **C.** Cell lysates 1×10^5 cells, were loaded on a 8% SDS PAGE gel, blotted and probed with Mab 4F3.

Role of 10-1 antigen in tumour growth and metastasis

Although 10-1 antigen expression on other rat carcinoma cell lines does not strictly correlate with metastasis, this fact alone does not mean that the antigen may not have a role in tumorigenesis. Tumour cells may acquire a number of properties which are not sufficient alone to trigger metastasis. For example, those non-metastatic cells lines that did express the 10-1 antigen perhaps had not yet acquired enough properties to become metastatic. On the other hand, metastatic cells which don't express the 10-1 antigen may have acquired other properties which obviate their need for 10-1 antigen expression. I therefore used the 10-1 antibodies in *in vitro* and *in vivo* experiments to determine their effect on the growth and metastatic spread of 10AS tumours.

I initially performed *in vitro* assays to consider the effect of the 10-1 antibodies on the proliferation rate of 10AS cells as measured by thymidine incorporation. Experiments were performed using both 2% and 10% FCS growth conditions. 10AS cells were cultivated in 2% and 10% FCS/RPMI together with either 5G8 (an antibody against CD44 that had been previously shown to have no anti-growth effects (Sleeman et al., 1996)), or with each of the 10-1 antibodies. After 48 hours incubation, ^{3}H-thymidine was added to 10μCi/ml. After a further 12 hour incubation the cells were transferred to filters using a Skatron cell harvester and the amount of incorporated thymidine measured by scintillation counting. Over the course of both experiment there was no dose-dependent effect on proliferation by the 10-1 antibodies. Even at the highest antibody concentrations no effect on proliferation of 10AS cells by the 10-1 antibodies was observed for cells grown in either 2% or 10% FCS/RPMI (Fig. 18 and Fig 19). These results show that the 10-1 antibodies in this tumour system have no direct effects *in vitro* on 10AS cell proliferation.

To test the effect of 10-1 antibodies on the growth of 10AS tumours in spontaneous metastasis assays, 10AS cells were injected subcutaneously into male and female BDX rats. The animals were treated over a period of 4 weeks with 5G8 and each of the 10-1 antibodies, as described in Materials and

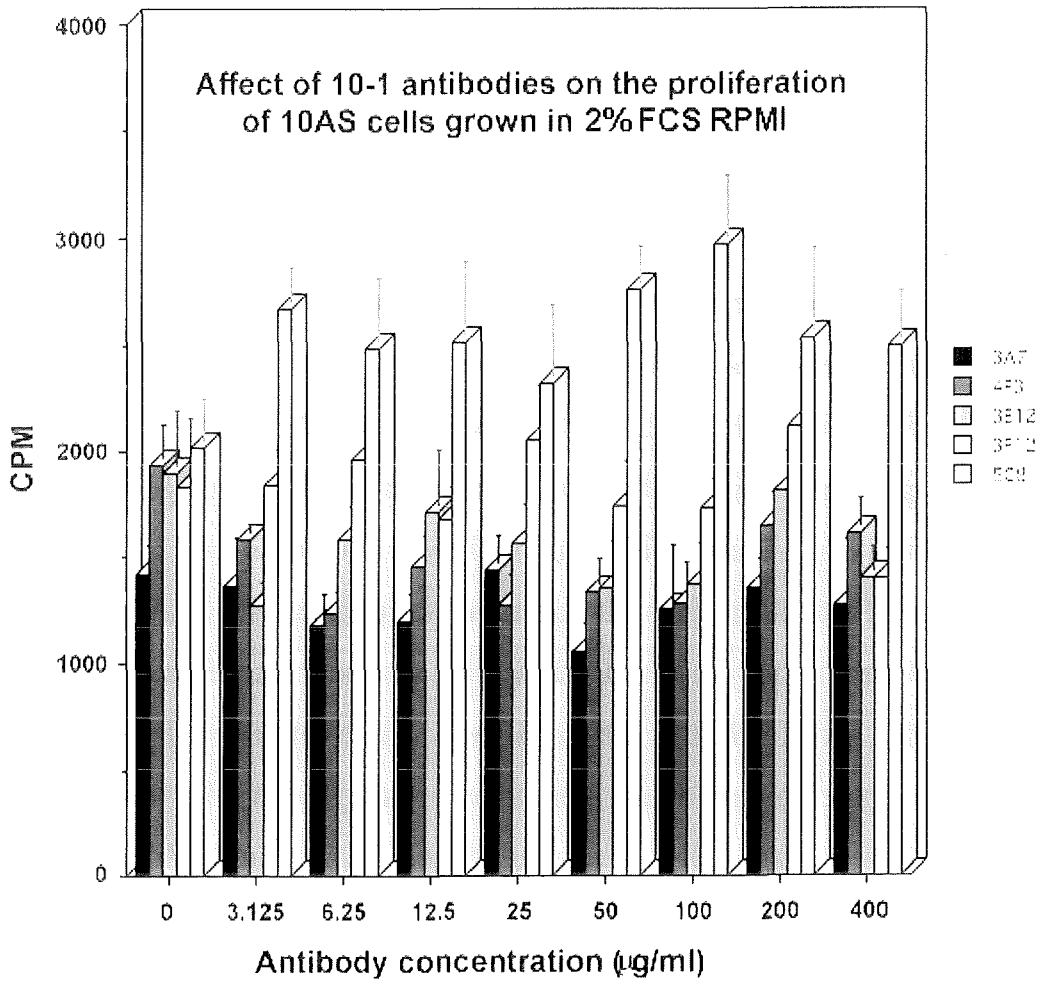


Figure 18. The effect of antibodies on the proliferation of 10AS tumour cells *in vitro*. 1×10^5 cells were cultivated with the appropriate antibody in a final volume of 100µl 2% FCS/RPMI and incubated for 2 days at 37°C, then ^{3}H-thymidine was added to 10 µCi/ml. After a further 12 hours incubation, the cells were harvested onto glass fiber filters with a Skatron Comi Cell Harvester and ^{3}H-thymidine uptake was assayed with a LKB Wallac liquid Scintillation Counter. Data is the average of quadruplicate experiments.

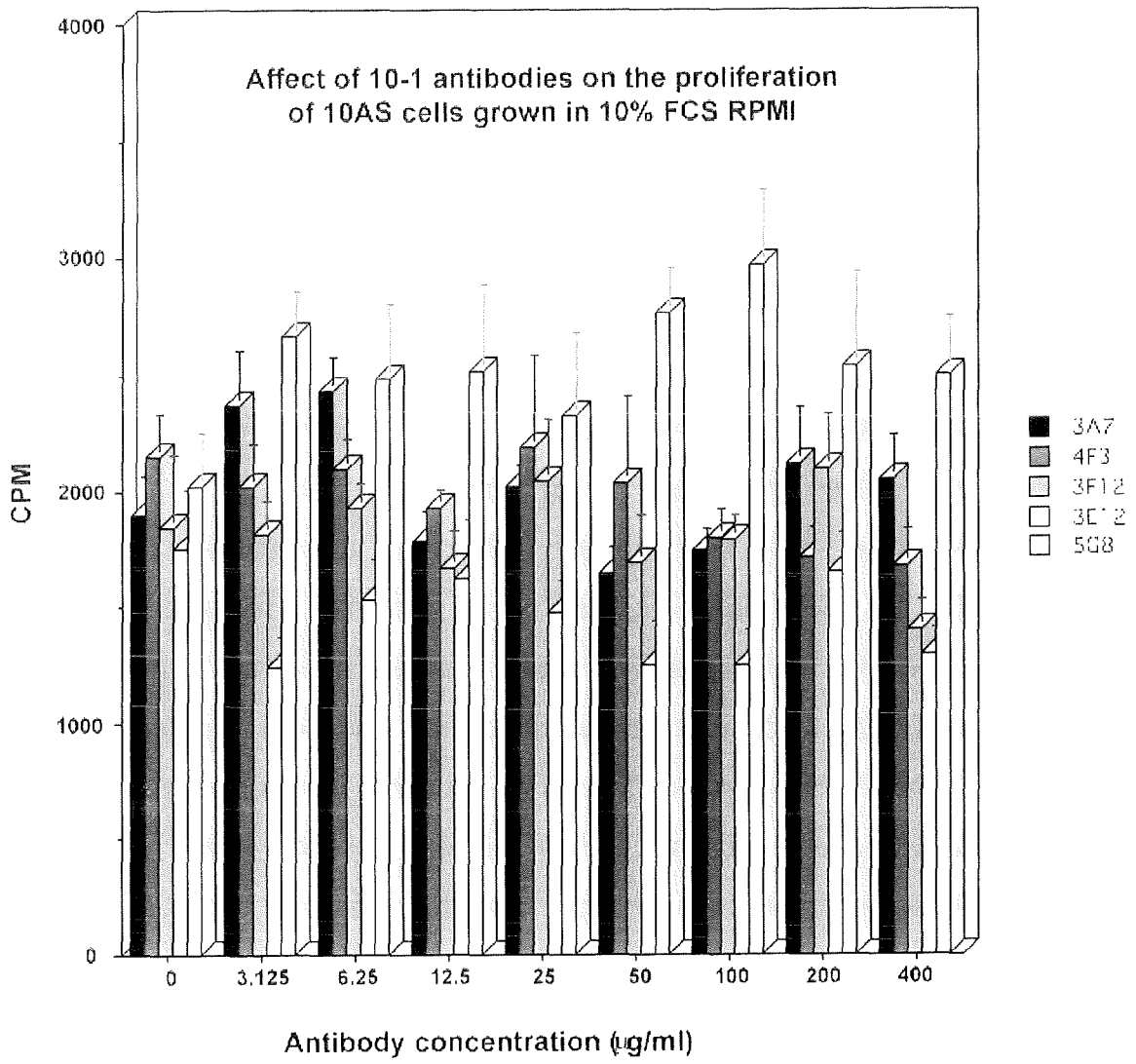


Figure 19. The effect of antibodies on the proliferation of 10AS tumour cells *in vitro*. 1×10^5 cells were cultivated with the appropriate antibody in a final volume of 100µl 10% FCS/RPMI and incubated for 2 days at 37°C, then ^{3}H-thymidine was added to 10 µCi/ml. After a further 12 hours incubation, the cells were harvested onto glass fiber filters with a Skatron Comi Cell Harvester and ^{3}H-thymidine uptake was assayed with a LKB Wallac liquid Scintillation Counter. Data is the average of quadruplicate experiments.

Methods. In each set of experiments, groups of 7 or 8 animals were treated with either 5G8 or the 10-1 antibodies. The 10-1 antibodies had a non-inhibitory effects on tumour growth or metastasis (Figure 20; Table 10). In all instances the treated animals died from growth of the injected tumour cells. There was a slight reduction in the number of animals having lymph node metastasis, but this was not statistically significant. These data demonstrate that the 10-1 antibodies do not inhibit tumour growth or metastasis.

At first sight, the observations from the *in vivo* experiments argue against the 10-1 antigen having a direct role in the tumour growth and metastasis of 10AS cells. However, there are arguments to suggest the contrary. Perhaps the 10-1 antibodies do bind the 10-1 antigen in *in vitro* and *in vivo* assays but have no inhibitory effect on the function of the 10-1 antigen. That is, they may recognise some structural feature of the 10-1 antigen that is only immunogenic in nature but has no direct functional role. Furthermore, I have also presented evidence that the 10-1 antigen is actively secreted by 10AS cells *in vitro*. Thus, any inhibitory effects of the antibodies may be nullified due to the antibodies binding to excess free antigen, for example. These arguments and the fact that the antigen is expressed by other rat carcinoma cell lines suggest that it could be associated with tumour growth and metastasis, but in a non-obligatory way which is alone insufficient. Thus the results of the functional assays with the 10-1 antigen are inconclusive. I therefore proceeded to purify the 10-1 antigen in order to microsequence it and thus determine its nature, with the hope of relating this to a function in metastasis.

Antigen purification

My attempts to identify a role for the 10-1 antigen in metastasis and tumour progression proved to be inconclusive. Thus to define a role and perhaps a function for this antigen, I needed to elucidate its identity. One approach could be to make a cDNA library from 10AS cells and make an

Table 10. Results of tumour inhibition experiments.

BDX rats were injected subcutaneously with 5×10^5 tumour cells in PBS. Thereafter, every 3 to 4 days 200µg monoclonal antibodies 3A7, 4F3, 3E12 or 3F12 were injected at the tumour site. Animals were regularly monitored until their tumours grew to the German legal limit or they became moribund, at which time they were killed and an autopsy performed.

Tumour +/- Mab	Tumour	Metastasis	Lymph node metastasis	Lung metastasis
10AS	8/8	8/8	8/8	1/8
10AS + 3A7	8/8	8/8	3/8	5/8
10AS + 4F3	7/7	7/7	7/7	2/7
10AS + 3E12	8/8	7/8	6/7	1/7
10AS + 3F12	7/7	7/7	5/7	4/7
10AS + 5G8	8/8	6/8	6/6	3/6

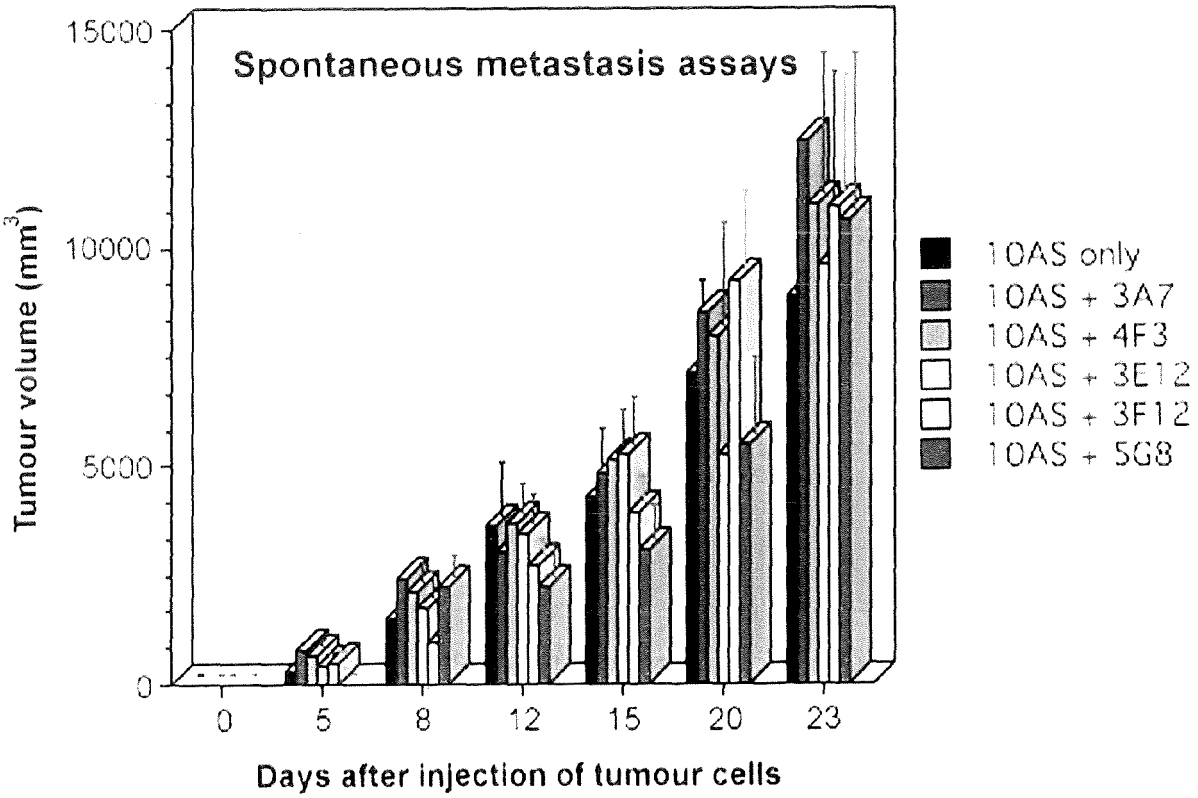


Figure 20. Primary tumour size of BDX rats subcutaneously injected with 5×10^5 10AS cells and treated with 5G8 control or 10-1 antibodies (see text for details of animals used). Tumour cells were injected in 100 µl PBS containing 200µg antibody. The rats were then treated with 200µg antibody each twice weekly for 4 weeks. Rats were killed when moribund or when the size of the primary tumour exceeded the German legal limit. Only rats in which tumours grew were included in the calculation of the average size of the primary tumour, to avoid introducting bias due to differences in the numbers of non-tumour bearing animals.

expression library. The 10-1 antibodies could then be used to probe for cDNAs encoding the 10-1 antigen. However, there are several problems with this approach. Firstly, the 10-1 antigen has multiple forms and it is not clear how these are related. Secondly, some of these forms are also weakly expressed in 1AS cells, meaning that cDNA clones purified out of such an expression library screen may not be those which are specific for 10AS cells. I therefore considered that the best biochemical approach for achieving this objective would be to purify the 10-1 antigen, using immobilised 10-1 antibodies to fish for the 10-1 antigen from cell lysates or conditioned media. The antibody-antigen bonds are then broken, allowing the antigen to be isolated and subjected to further treatment to reach sufficient concentration and purity for N-terminal sequencing to be performed. The derived N-terminal sequence can then be compared to protein sequences from a variety of data bases to find its identity, or can be used to make oligonucleotide probes to make cDNA libraries.

Preparation of a 10-1 antigen affinity column

I set out to use immunoaffinity purification to purify the 10-1 antigen from 10AS cell lysate, or conditioned medium. Antibodies were bound to protein G agarose beads (Dianova) and cross-linked to the beads with dimethylpimelimidate (DMP; protein G-DMP-Mab), as described in Materials and Methods. Protein G beads were used because they have a greater affinity for IgG_1 and IgG_3 antibodies than protein A beads (Harlow and Lane, 1988). The crosslinking of the antibodies to the protein G beads was checked by boiling the beads in reducing sample buffer, subjecting the supernatants to electrophoresis and staining the gel with coomassie blue to check that DMP had efficiently bound all Mabs to the protein G beads (Fig. 21).

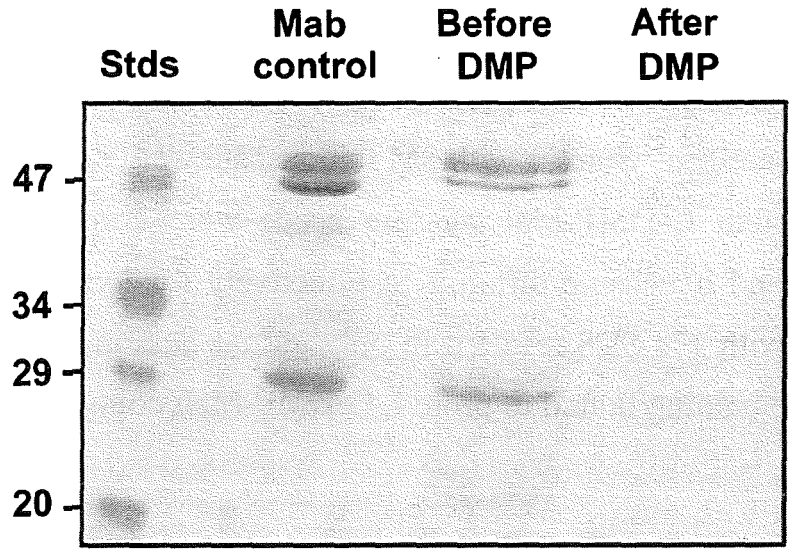


Figure 21. Checking of crosslinked protein G-DMP-Mab columns. Columns were prepared as described in Materials and Methods. Beads were taken before and after the DMP reaction boiled in reducing sample buffer, eluate loaded on a 10% SDS PAGE gel, electrophoresis performed and the gel coomassie stained. Mab control shows migrating heavy (47-50 kDa) and light (28-30 kDa) chains. Note that in the After DMP reaction no Mabs are present.

Identification of conditions for eluting the 10-1 antigen

Cell lysates and dialysed conditioned media (DCM) were prepared as described in Materials and Methods and precleared with protein G beads for 4 hours or overnight at 4°C, after which the DCM was incubated overnight at 4°C with protein G-DMP-3E12 beads. In initial experiments small scale preparations were performed to optimise antigen elution conditions. Protein G-antibody-antigen complexes were washed in PBS and then incubated with one of following reagents: low pH (100mM glycine pH 2.5), high pH (100mM HCO_3), high salt (5M LiCl), low pH high salt (100mM glycine pH 2.5, 5M LiCl), high pH high salt (100mM HCO_3 pH 11.5, 5M NaCl), 1% SDS, 2M urea, 8M urea, 2M guanidine HCl, 3M thiocyanate or 10% dioxane, to test for their ability to elute the 10-1 antigen. The eluates (E) and the treated beads (B) were subjected to SDS PAGE electrophoresis, blotted and probed with the 10-1 antibodies (Fig. 22A and B). The effectiveness of each reagent was judged by comparing E and B for 10-1 antigen retention. Protein G-DMP-Mab beads

with or without bound antigen where used as controls. In these experiments 3M thiocyanate caused complete elution and 1% SDS caused minimal release of the antigen from protein G-DMP-Mab beads. None of the other reagents eluted any 10-1 antigen. Studies with 3M thiocyanate were not pursued further because the protein G-DMP-Mab agarose beads were completely destroyed during elution and it became difficult to effectively remove the eluate (Fig. 22B). Elution with 1% SDS resulted in only partial elution with the majority of the antigen remaining bound to protein G-DMP-Mab columns (Fig. 22B).

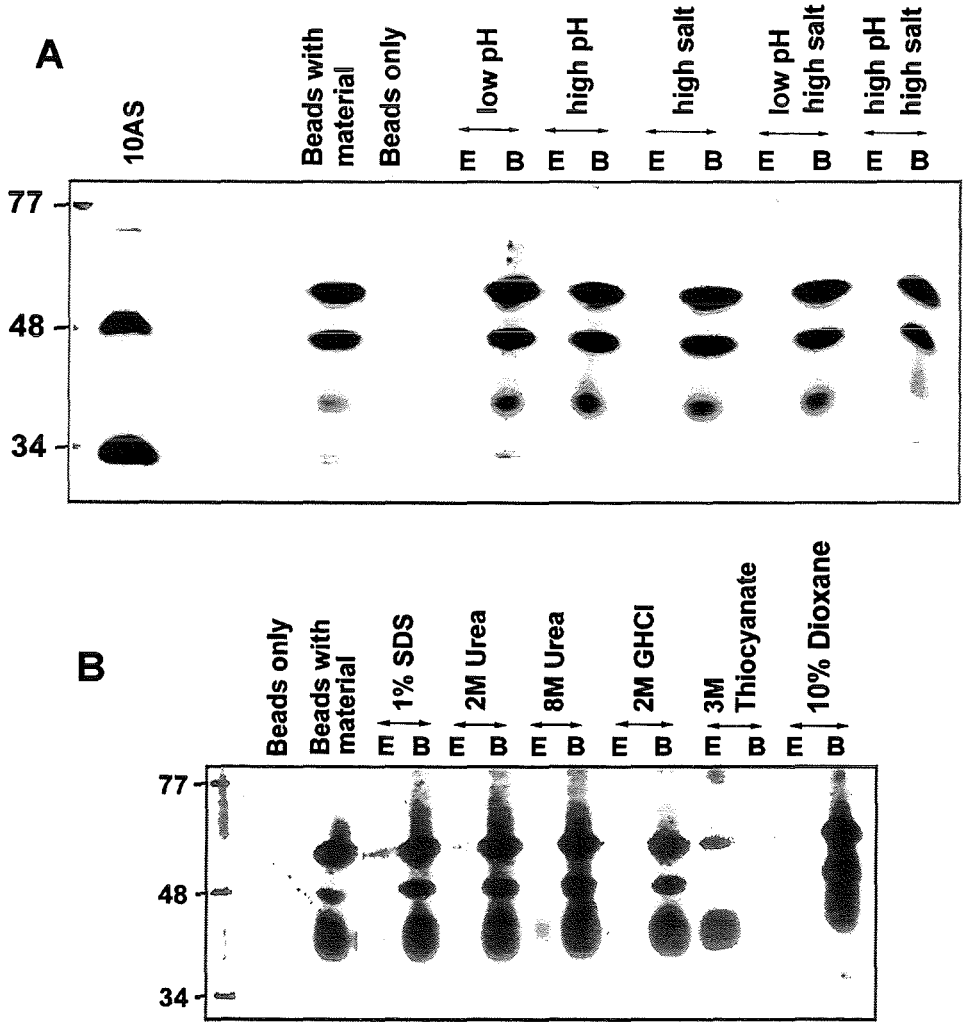


Figure 22A and B. Establishment of elution conditions. Protein G precleared 10AS dialysed conditioned medium was incubated overnight with protein G-DMP-3E12 beads. The beads were washed in PBS and treated with low pH (100mM glycine pH 2.5), high pH (100mM HCO_3), high salt (5M LiCl), low pH high salt (100mM glycine pH 2.5, 5M Li Cl), high pH high salt (100mM HCO_3 pH 11.5, 5M NaCl), 1% SDS, 2M urea, 8M urea, 2M guanidine HCl, 3M thiocyanate or 10% dioxane. The eluates (E) and the treated beads (B) were boiled in non-reducing sample buffer, subjected to SDS PAGE electrophoreses, blotted and probed with the 10-1 antibodies.

Purification of the 10-1 antigen from cell lysates

In order to optimise the elution by SDS, I decided to boil the beads for 5 min in 1% SDS to try to break the antigen-antibody bonds more effectively. After boiling, the eluate was removed. The beads were then boiled again in non-reducing sample buffer, to check for the efficiency of elution. The boiled SDS eluate was also subjected to various further treatments to improve the purity and concentration of the eluted 10-1 antigen (Fig. 23). Thus the eluate was concentrated by two centrifugal concentration steps through AMICON Centricon concentrators. The eluate was firstly applied to a Centricon-100 concentrator to remove all proteins above 100 kDa, and the flow through containing the 10-1 antigen then concentrated to a minimal volume by a Centricon-30 concentrator (30 kDa cut-off) for electrophoretic analysis. At each stage of the purification process samples were taken and subjected to SDS PAGE electrophoresis and western blotting. To consider if antibodies had leached from protein G-DMP-Mab beads, the gels were blotted and probed separately with either the 10-1 antibodies and secondary HRP antibody, or the secondary HRP antibody alone. The majority of the purified eluate was applied to a separate lane on the same SDS PAGE gel. After blotting, the lane corresponding to this sample was cut from the PVDF membrane and stained with coomassie blue to visualise if there was sufficient puried protein for N-terminal sequencing analysis.

Figure 24 is the result of an experiment using this protocol with 9.0×10^7 cells. It demonstrates that elution of the 10-1 antigen from protein G-DMP-3E12 beads by 1% SDS is much more efficient after boiling. From this experiment with cell lysate a number of conclusions can be made. The form of 10-1 antigen which was eluted from the protein G-DMP-3E12 beads migrates as a major band of 55-60 kDa and a minor band of 34-36 kDa (Fig. 24, lane C). These bands correspond to those recognised by the 10-1 antibodies in 10AS cell lysate (Fig. 24, Lane G). There is non-specific protein binding to

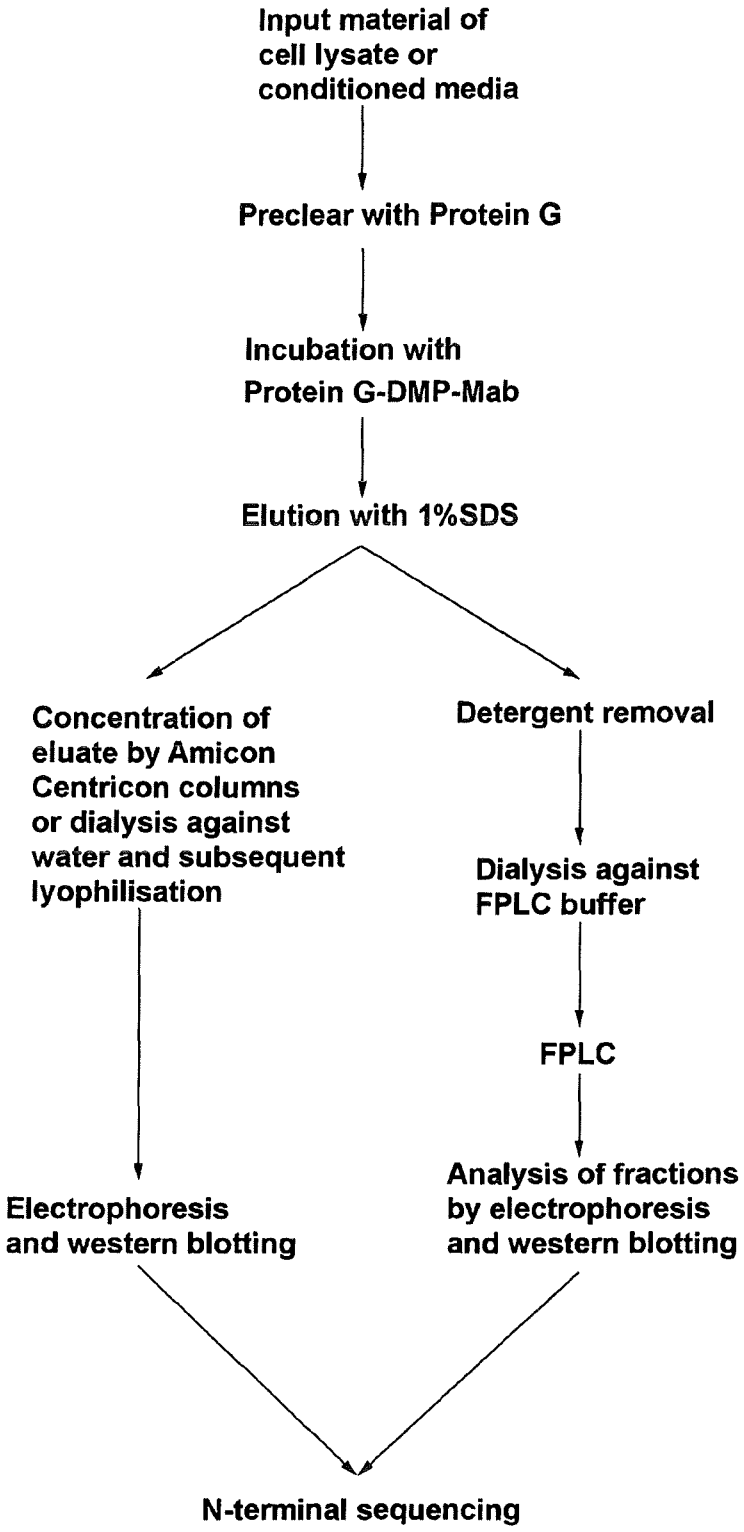


Figure 23. Flow diagram of 10-1 antigen purification. The eluates were either concentrated or further processed for FPLC analysis, prior to being subjected to electrophoresis and western blotting to examine 10-1 antigen content.

protein G beads migrating at 40 kDa and 55 kDa, which is probably immunoglobulin heavy chain recognised by the secondary antibody (Fig. 24, Lane C). The 10-1 antigen is not present in the flow through of the Centricon 100 concentrator in both experiments (Fig. 24, lane D), suggesting the 10-1 antigen either binds to the membrane of the concentrator or is still bound to leached 3E12 antibody leading to a total molecular weight of over 100 kDa, and is therefore not permitted to pass through the concentrator's membrane. The protein G-DMP-3E12 beads after elution have residual 10-1 antigen attached, suggesting the 1% SDS elution conditions are not totally effective, resulting in a reduced yield of purified antigen. These data suggested that elution with 1% SDS is not complete and that the majority of the eluted antigen remains attached to antibodies that leached from the protein G-DMP-3E12

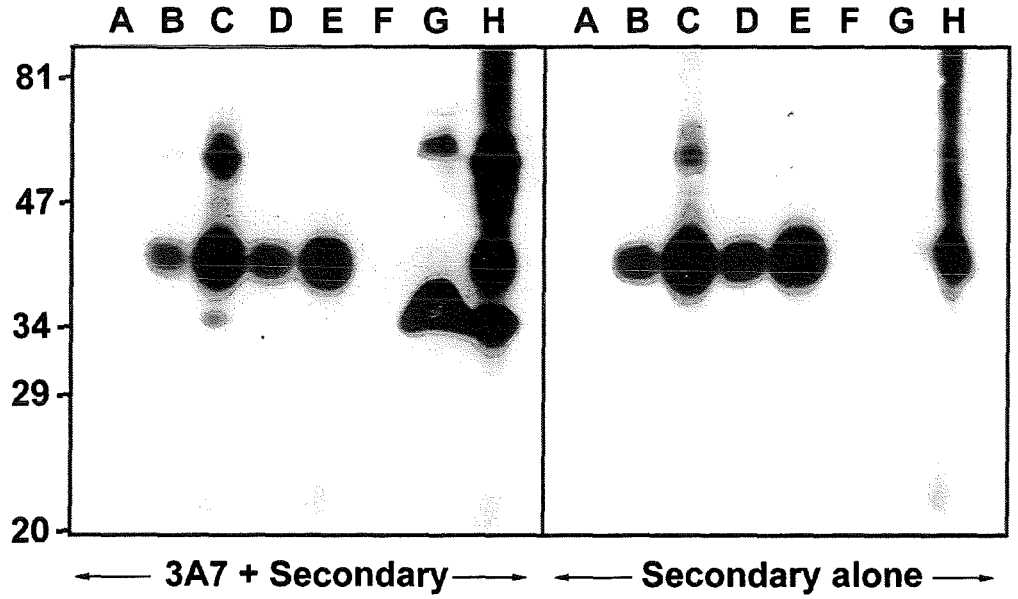


Figure 24. Purifications trial with 10AS cell lysate. 9.0×10^7 cells were lysed in 1% NP40 PBS containing 2mM PMSF. The lysate was precleared with protein G and then incubated overnight with protein G-DMP 3E12 beads. Elution of 10-1 antigen was performed with the addition of 1% SDS PBS and incubated at 100°C for 5 min. The eluate was dialysed in 50mM Tris pH 9.0, the detergent removed, concentrated by an AMICON Centricon 100-concentrator and the flow through collected and concentrated by an AMICON Centricon-30 concentrator. An aliquot from each purifcation step was loaded onto a SDS PAGE gel in the following order: **A**, protein G-DMP 3E12 beads; **B**, elution; **C**, supernatant from Centricon-100 concentrator **D**, flow through from Centricon-100 concentrator; **E**, supernatant Centricon-30 concentrator; **F**, flow through from Centricon-30 concentrator; **G**, 10AS RIPA cell lysate; **H**, protein G-DMP 3E12 beads after elution, electrophoresed, blotted and probed with the 3A7 Mab and secondary HRP antibody, and secondary HRP antibody alone.

beads. These data also demonstrate the high affinity of the 10-1 antibodies for the 10-1 antigen, as boiling in 1% SDS is an extremely harsh elution.

Attempts to increase 10-1 antigen elution

To attempt to improve the release, quantity and purity of the 10-1 antigen from protein G-DMP-3E12 beads, elutions were performed with 2% SDS with and without reduction. The eluate was concentrated only once with an AMICON Centricon-10 concentrator to limit loss of the 10-1 antigen during the concentration steps. As a prelude to purifications performed with FPLC where the efficiency of certain chromatography procedures are effected by detergents, a detergent removal step was added to test how much 10-1 antigen yield would be affected. Antigen purification was tracked by taking equivalent aliquots at each purification step and subjecting them to electrophoresis under non-reducing and reducing conditions. Electrophoresis was performed on a 30cm long SDS PAGE gel to allow better separation of the proteins and thus potentially attain a cleaner signal for N-terminal sequence analysis.

Figures 25 and 26 show the results of 10-1 antigen purification with 2% SDS with boiling under non-reducing and reducing electrophoretic conditions, respectively. To remove non-specific binding proteins from the purification process, the cells lysates were first incubated for a minimum of two hours with protein G beads. An amount of the 10-1 antigen present in the cell lysates bound non-specifically to the protein-G beads. This can be visualised by comparing lanes B and C, of Fig. 25A and Fig. 26A. In both experiments the protein G-DMP-3E12 beads had a high affinity for the 10-1 antigen, removing the majority of it from the cell lysate (compare lanes C and D, Fig. 25A and 26A). However, again the efficiency of elution with 2% SDS was poor, resulting in a large amount of 10-1 antigen remaining attached to the protein G-DMP-3E12 beads (compare lanes F and H, Fig. 25A; and lanes H and J, Fig. 26A). Thus it appears that despite changing the elution conditions to 2% SDS the release of the 10-1 antigen by the 10-1 antibodies is unaffected. Therefore

the major deficiency in 10-1 antigen purification is the initial breakage of the antigen-antibody bonds.

Detergent removal was tested only for the purification under reducing conditions (Fig. 26). It was found that removal of SDS detergent had little effect on 10-1 antigen yield (Fig 26A, compare lanes F and G). Concentration of the eluate by Amicon Centricon-10 concentrators resulted in a reduction of antigen yield. In Fig. 25A, lanes F and E are of similar intensity, and for Fig. 26A lane H is 3 to 4 times more intense than lane G, whereas the process

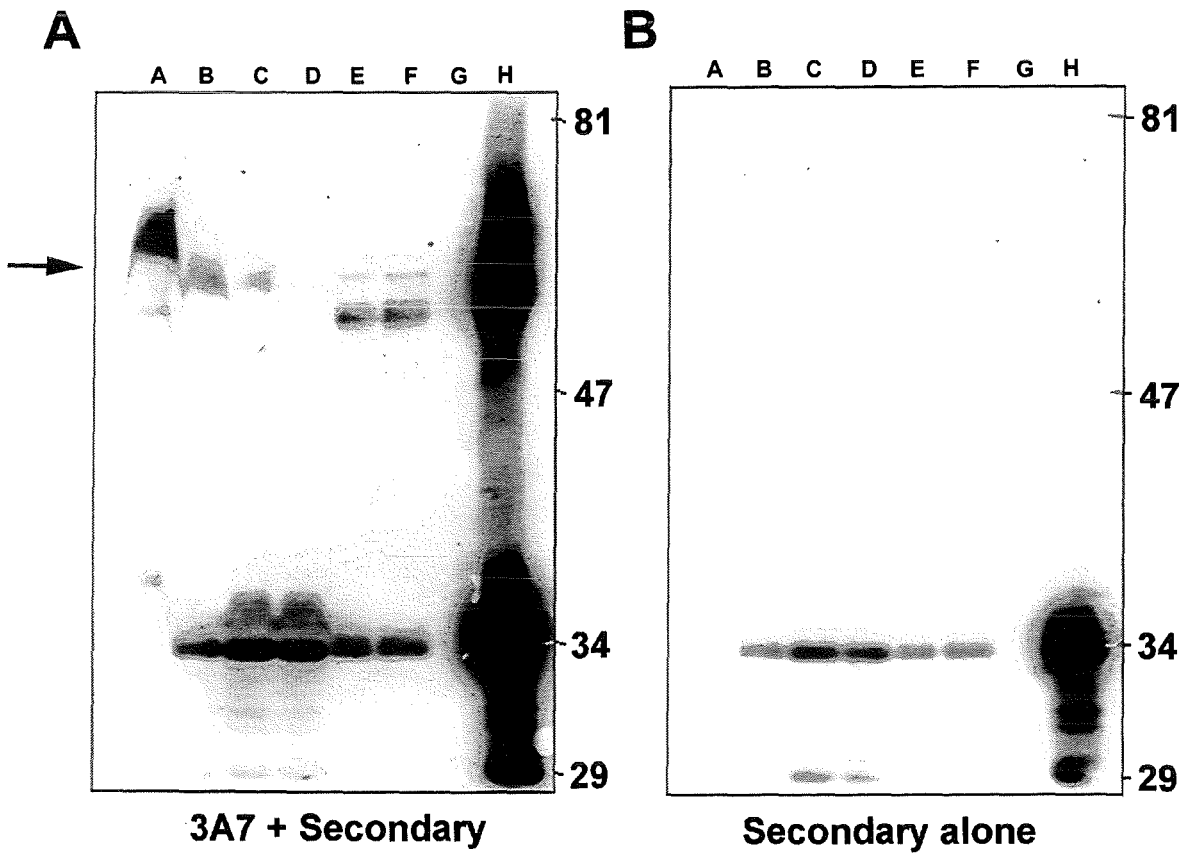


Figure 25A and B. SDS PAGE gel of 10-1 antigen purification by 2% SDS performed under non-reducing conditions. 2.0×10^9 cells were lysed in 1% NP40 PBS containing 2mM PMSF. The lysate was precleared with protein G and then incubated overnight with protein G-DMP-3E12 beads. Elution of 10-1 antigen was performed with the addition of 2% SDS PBS and incubated at 100°C for 5 min. The eluate was dialysed in 50mM Tris pH 9.0, the detergent removed and concentrated by a Centricon 10 concentrator. An aliquot from each purification step was loaded onto a SDS PAGE gel in the following order: **A**, 10AS cell lysate; **B**, NP40 10AS cell lysate; **C**, after pre-clear with protein G; **D**, after incubation with protein G-DMP-3E12 beads; **E**, elution; **F**, supernatant from Centricon-10 concentrator; **G**, flow through from Centricon-10 concentrator; **H**, protein G beads after elution, electrophoresed on a 30 cm long gel, blotted and probed with the 3A7 Mab and secondary HRP antibody (Fig. 25A) and secondary HRP antibody alone (Fig. 25B). The arrow corresponds to the migration of the 10-1 antigen.

should have resulted in a 10-fold concentration, demonstrating that a large quantity of the 10-1 antigen has been lost during the concentration process.

Blots made from gels identical to those in Figures 25A and 26A were probed with the secondary antibody alone to detect any antibodies that may have had leached from the protein G-DMP-3E12 beads (Fig. 25B and 26B). As a control, 2μg 3A7 Mab was loaded on the reducing gel (Fig. 26B, lane K) and was recognised by the secondary-HRP-labeled antibody as heavy chain 48 kDa and light chain 34 kDa components. Also detected by the secondary HRP antibody were non-specific proteins and light chain molecules migrating between 28-36 kDa. Lanes G and H of Fig. 26B have more intense bands in

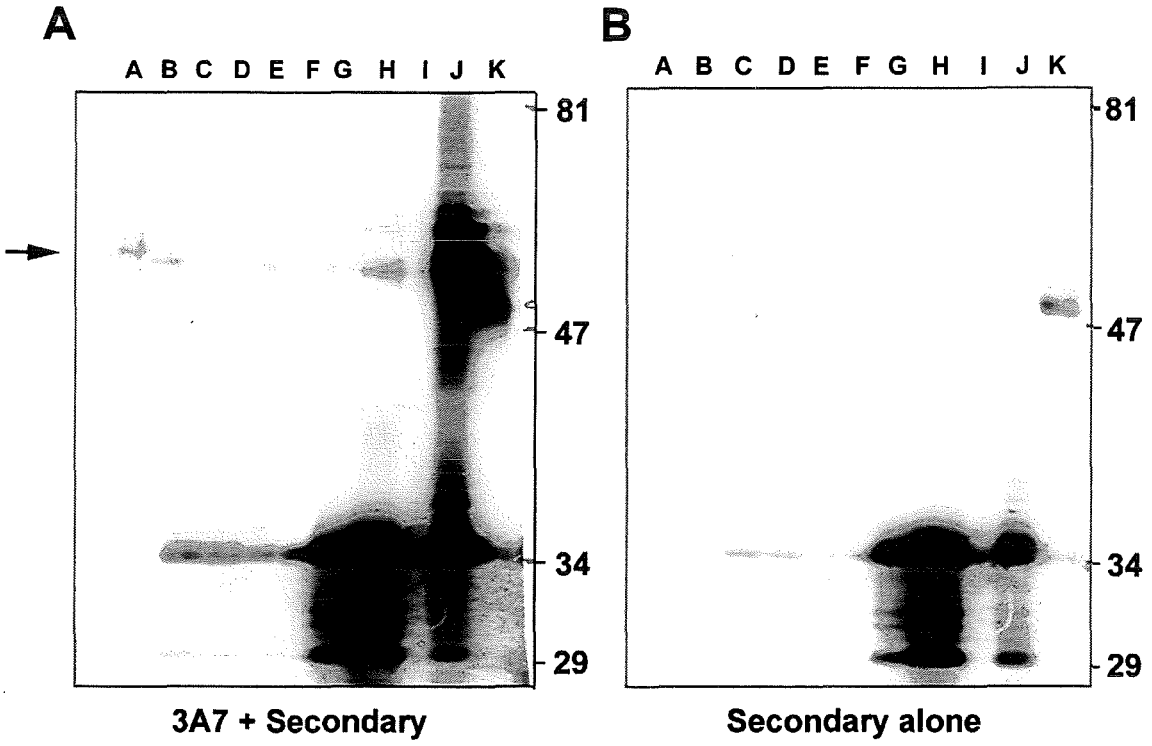


Figure 26A and B. SDS PAGE gel of 10-1 antigen purification by 2% SDS under reducing conditions. 2.0×10^9 cells were lysed in 1% NP40 PBS containing 2mM PMSF. The lysate was precleared with protein G and then incubated overnight with protein G-DMP-3E12 beads. Elution of 10-1 antigen was performed with the addition of 2% SDS PBS and incubated at 100°C for 5 min. The eluate was dialysed in 50mM Tris pH 9.0, the detergent removed and concentrated by a Centricon 10 concentrator. An aliquot from each purification step was loaded onto a SDS PAGE gel in the following order: **A**, 10AS cell lysate; **B**, NP40 10AS cell lysate; **C**, after pre-clear with protein G; **D**, after incubation with protein G-DMP-3E12 beads; **E**, elution; **F**, after detergent column; **G**, after pre-clear with protein G; **H**, supernatant from Centricon-10 concentrator; **I**, flow through from Centricon-10 concentrator; **J**, protein G beads after elution; **K**, 2μg 3A7 Mab; and electrophoresed on a 30 cm long gel, blotted and probed with the 3A7 Mab (Fig. 26A) and secondary HRP antibody and secondary HRP antibody alone (Fig. 26B). The arrow corresponds to the migration of the 10-1 antigen.

this molecular weight range than lanes E and F of Fig. 25 B, which is probably due to more light chains being cleaved from antibody molecules by the action of DTT present in the reducing sample buffer. The remaining concentrated 10-1 antigen was also applied to the same gel in each experiment and blotted. This lane was separated from remaining PVDF membrane used for western blot analysis, and stained with coomassie blue according to the manufacture`s instructions. In both experiments a protein band migrating in the range of 55-65 kDa corressponding to the 10-1 antigen was not visible. These results demonstrate that this elution and purification protocol does not efficiently remove the 10-1 antigen from the protein G-DMP-antibody beads and what antigen is removed is contaminated by non-specific-binding proteins and antibody components.

To attempt to improve elution of the 10-1 antigen 1% SDS 100mM DTT with boiling was used to break 10-1 antigen antibody bonds and a similar purification process as described above was used (data not shown). This methods gave the best yield of eluted 10-1 antigen as evident by bands on western blots and on the PVDF coomassie stained membrane. However, several other proteins migrated within the same region as the 10-1 antigen, resulting in a noisy signal when subjected to N-terminal sequencing. No distinct amino acid sequence was obtainable.

FPLC purification of 10-1 antigen eluates

Having observed from the above results that several proteins were purified along with the 10-1 antigen, I decided to use a Pharmacia FPLC chromatography system to isolate the 10-1 antigen from affinity purified eluates. The antigen was eluted by boiling with 1% SDS containing 100mM DTT. The eluate was dialysed against 50mM Tris, pH 9.0 and the detergent removed by Extracti-Gel® D Detergent Removing Gel. The eluate was then dialysed against the respective chromatography buffer and incubated for

Figure 27

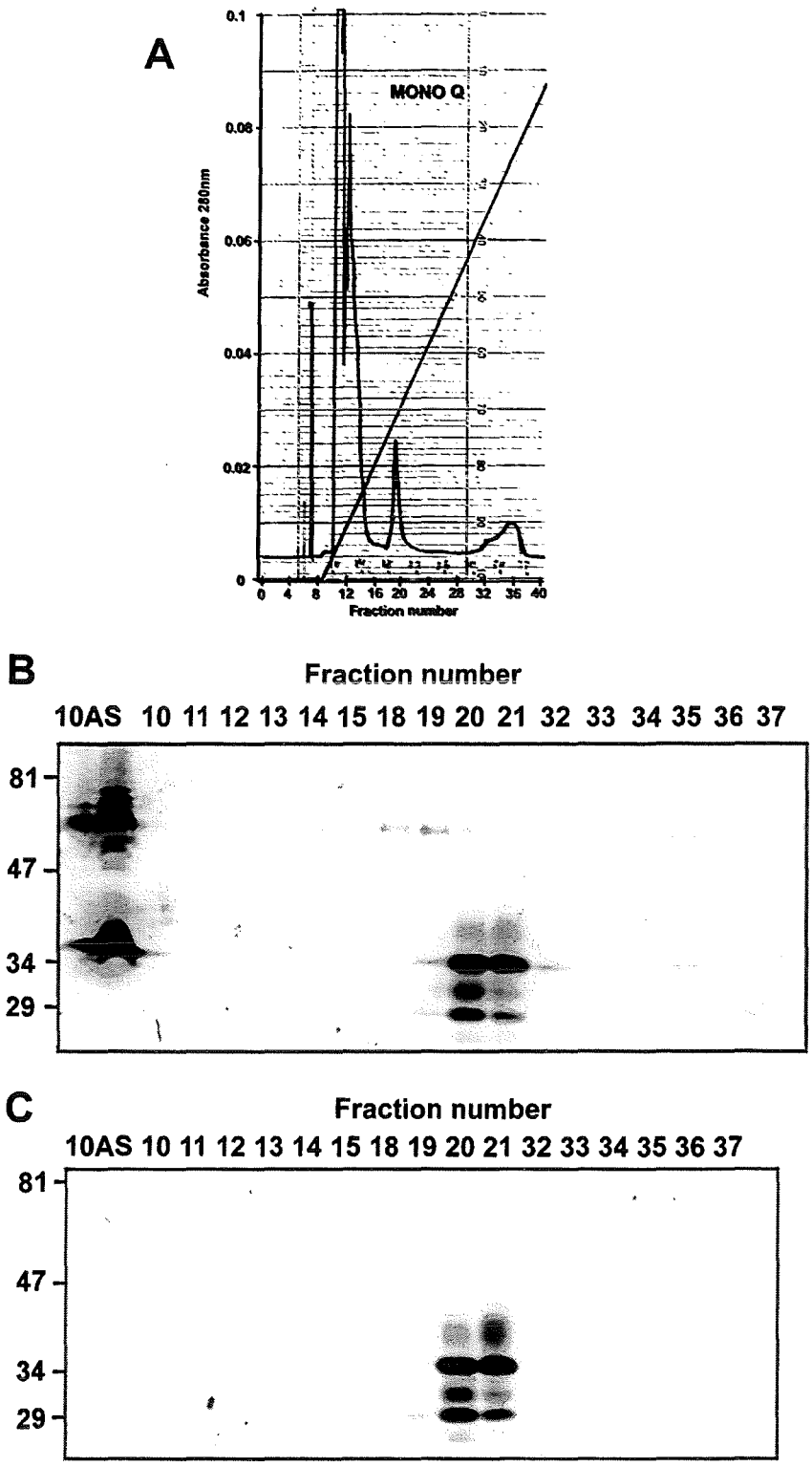
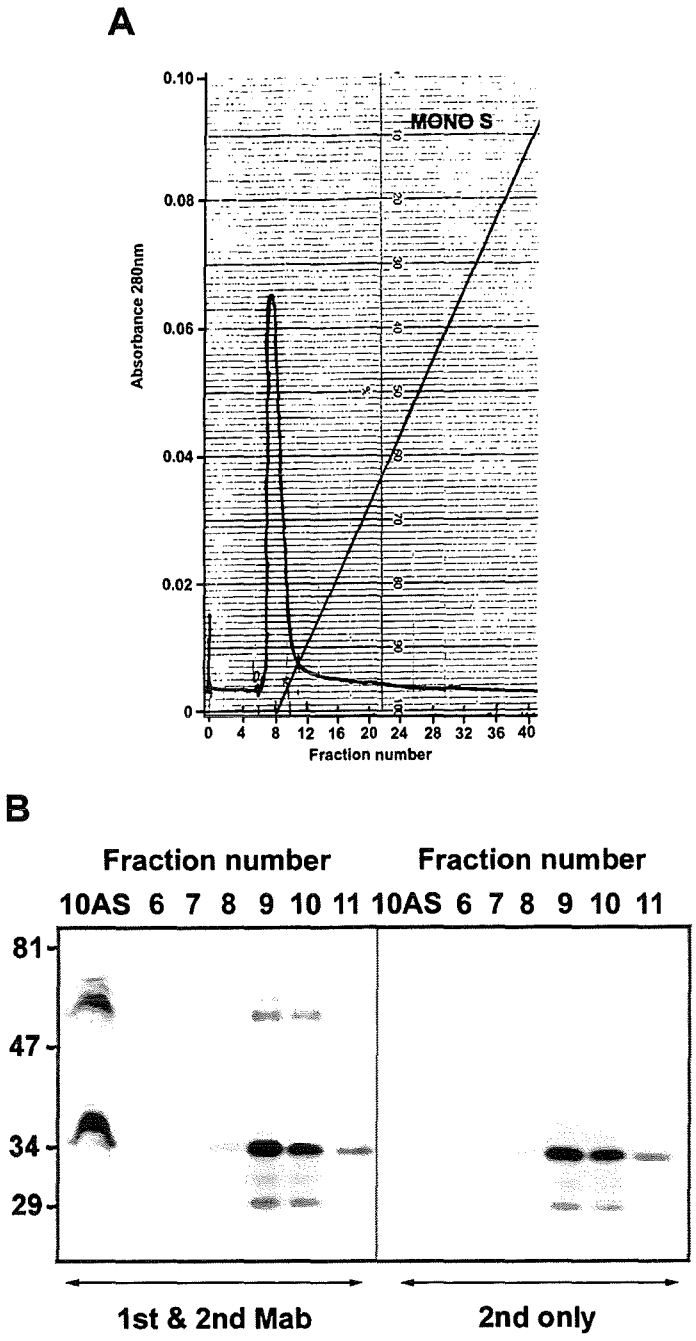

A
MONO Q
Absorbance 280nm
Fraction number
B
Fraction number
10AS 10 11 12 13 14 15 18 19 20 21 32 33 34 35 36 37
81
47
34
29
C
Fraction number
10AS 10 11 12 13 14 15 18 19 20 21 32 33 34 35 36 37
81
47
34
29

Figure 28



Figures 27 and 28. FPLC purification of 10-1 antigen. 8.6×10^8 cells/experiment were lysed in 1% NP40, precleared with protein G, incubated overnight with protein G-DMP-3E12 beads, eluted with 1% SDS 100mM DTT, dialysed against 50mM Tris, pH 9.0, the detergent removed with Extracti-Gel® D Detergent Removing Gel, and dialysed against the respective FPLC buffer. A Pharmacia FPLC chromatography system was utilised with Mono Q (Fig. 27A) and Mono S (Fig. 28A) columns. The eluting buffers were 20mM Tris, 1M NaCl, pH 7.5 (for Mono Q) and 50mM Phosphate Buffer, 1M NaCl, pH 7.0 (for Mono S). Chromatography was performed at a flow rate of 1ml/min. For analysis, the sample was injected onto the column in the respective dialysis/binding buffer at 3 min and at 5 min the eluting buffer was applied to the column in the form of a linear gradient, with its concentration increasing from 0% to 100% over 20 minutes. Two 0.5ml fractions/minute were collected and analysed continuously for UV absorbance. Fractions corresponding to peaks of UV absorbing material on the printed chromatogram were subjected to SDS PAGE electrophoresis, blotted and probed with 3A7 Mab and secondary HRP antibody (Fig. 27B and 28B) and secondary HRP antibody alone (Fig. 27C and 28B).

two hours with protein G beads to remove any immunoglobulins that may have leached from the affinity column. The final product was applied to either a Mono Q or Mono S chromatograpy column and chromatography performed as described in Materials and Methods. The fractions corresponding to peaks of UV absorbing material on the chromatogram were further analysed for the presence of the 10-1 antigen. The large peaks in Fig. 27, fractions 10-14 and Fig. 28, fractions 6-10, correspond to detergent absorbance, due to the aromatic ring present in NP40. Analyses by western blot showed that the 10-1 antigen was indeed purified (Figs. 27 and 28). Mono Q (anion exchanger) gave the best purification (Fig. 27), separating the 10-1 antigen from proteins recognised by the secondary antibody alone. The 10-1 antigen migrated at 60-65 kDa and was present in highest quantities in fractions 18 and 19 and also in low quantities in fractions 34 to 36. Purifications with Mono S (cation exchanger, Fig. 28) resulted in the antigen passing directly through the column and being grouped with eluted detergent and molecules recognised by the secondary HRP antibody. These data suggest that at pH 7.0 the protein has a net negative charge and as such only binds to the anion exchange column, Mono Q. Fractions from both chromatography columns which had the highest quantity of the 10-1 antigen, namely, fractions 18 and 19 from Mono Q and fractions 9 and 10 from Mono S, were concentrated and subjected to SDS PAGE electrophoresis and the gel silver stained (data not shown). No protein corresponding to to the molecular weight of the 10-1 antigen could be seen after silver staining, suggesting that the chromatography procedure had the detrimental effect of further reducing the end yield. As I had used a substantial quantity of cells for this purification procedure (8.6×10^8 cells), it seemed unlikely that increasing the quantity of starting material could result in an yield improvement. I therefore abandoned this approach and chose to concentrate on another procedure.

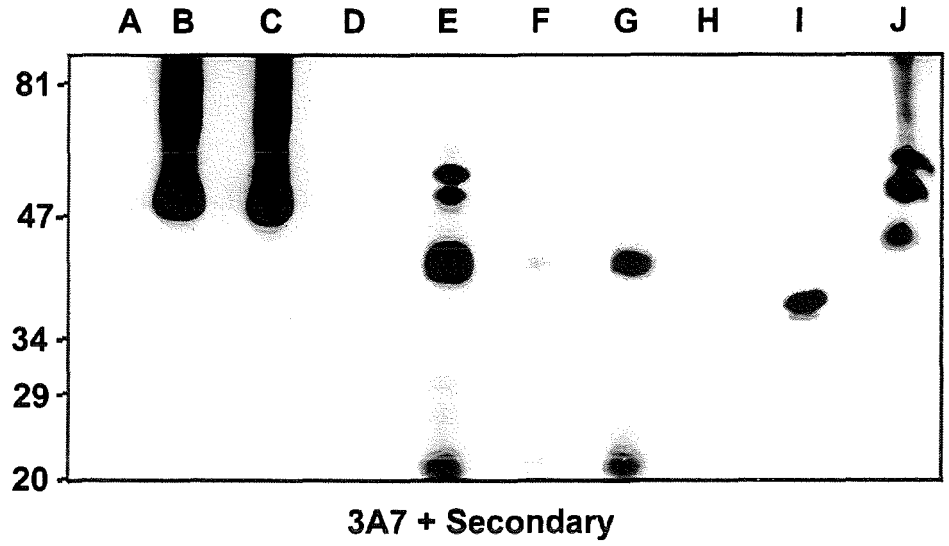
Purifications of 10-1 antigen from conditioned media

Conditioned medium contains a lower complexity of protein material than cell lysate and therefore purifications from it should contain less proteins capable of non-specifically binding to the affinity columns. Knowing that the 10-1 antigen is secreted from 10AS cells I decided to attempt 10-1 antigen purifcation utilising conditioned media from 10AS cells.

40 ml of conditioned media was prepared as described in Materials and Methods, precleared overnight with protein G and further incubated overnight with protein G-DMP-3E12 beads. The antigen was eluted with 1% SDS by boiling for 5 minutes and the eluate applied to a AMICON Centricon-100 concentrator to remove unwanted and non-specific proteins above 100 kDa. The flow through from the Centricon-100 concentrator was further concentrated by application to a AMICON Centricon-30 concentrator. An aliquot from elution, supernatants and flow throughs from each concentration step were loaded onto a SDS PAGE gel, blotted and probed with the 10-1 antibodies and secondary HRP antibody for antigen detection, and the secondary HRP antibody alone for antibodies that may have leached from the protein G-DMP-3E12 beads (Fig. 29). To take into consideration any non-specific binding, controls were run of protein G-DMP-3E12 beads incubated with RPMI and RPMI plus 10% FCS.

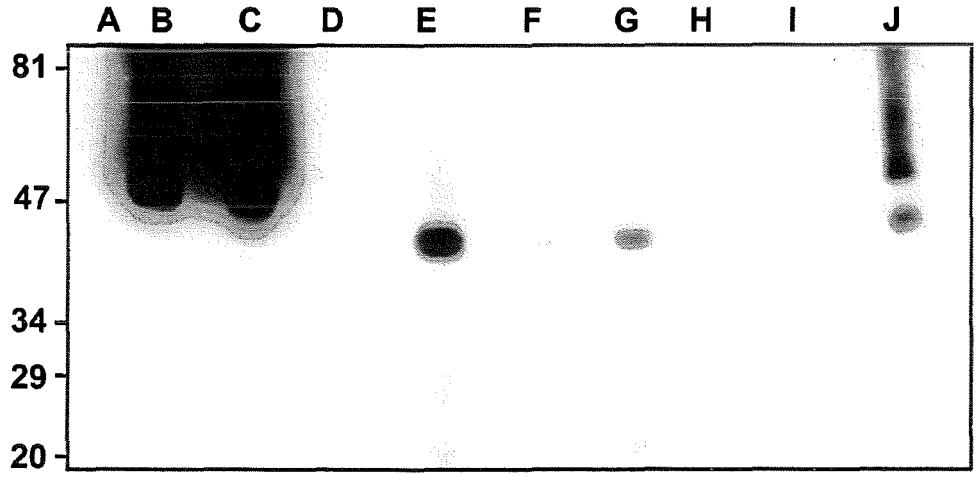
The most obvious feature of this experiment was the reduced non-specific protein-binding in the elution (Fig. 29A, lane D) and concentrated fractions (Fig. 29B, lane E and G). The observed non-specific protein binding to protein G-DMP-3E12 beads was due to material present in the FCS and is visualised as a broad smear from 47-85 kDa (Fig. 29, lanes B and C). Non-specific protein-binding was also present in the concentrated supernatants and migrated in the range 20-34 kDa and as a single band of 40 kDa which appeared after concentration (Fig. 29, lanes E, F and G). The majority of non-specific binding-proteins migrating from 47-85 kDa were removed after the washing of the protein G-DMP-3E12 beads before elution. Only the antigen

A



3A7 + Secondary

B



Secondary alone

Figure 29A and B. Purification trial with 10AS conditioned media. 40ml conditioned media was precleared with protein G and the supernatant incubated with protein G-DMP-3E12 beads overnight. Elution of 10-1 antigen was performed with the addition of 1% SDS PBS and 5 min incubation at 100°C. The eluate was passed through a Centricon 100 concentrator and the flow through concentrated by application to a AMICON Centricon-30 concentrator. An aliquot from each elution step was loaded onto a non-reducing SDS PAGE gel in the following order: **A**, RPMI; **B**, RPMI + 10% FCS; **C**, DCM; **D**, elution; **E**, supernatant Centricon-100 concentrator; **F**, flow through Centricon-100 concentrator; **G**, supernatant Centricon-30 concentrator; **H**, flow through Centricon-30 concentrator; **I**, 10AS cell lysate; **J**, protein G beads after elution; electrophoresed, blotted and probed with the 3A7 Mab and secondary HRP antibody (Fig. 29A) and secondary HRP antibody alone (Fig. 29B).

and non-specific binding-proteins of 20-34 kDa and 40 kDa remained after the elution and concentration steps.

This experiment was performed in conjunction with the initial purifications performed with cell lysates (Fig. 24). I thus employed the same eluate concentration process of Amicon Centricon-100 and -30 columns. Therefore, I encountered the same problems of 10-1 antigen loss, due to it binding to the concentrators membrane or being unable to pass through the membrane because it is still bound to an antibody molecule (compare lanes E and F, and G and H, Fig. 29A). I tried to overcome the latter problem by supplementing the elution with 100mM DTT to break 10-1 antigen-antibody complexes, allowing the 10-1 antigen to be concentrated (data not shown). However in these experiments the 10-1 antigen remained largely in the supernatant of the concentrators, suggesting that some other chemical property, such as binding of the 10-1 antigen to the concentrators membrane had reduced the end yield. In light of these observations, for future experiments I reduced yield loss by dialysing the eluate against water and performing lyophilisation and taking the lyophilised 10-1 antigen product up in a smaller volume of buffer. It should be noted that I employed this approach for elutions with cell lysates and attained a greater end yield as seen on western blots, but increased non-specific protein-binding was also observed which interfered with N-terminal sequencing attempts (data not shown).

Large scale purification of 10-1 antigen from conditioned media

After considering the results of the experimental approaches that I had used, I decided that the best method to purify the 10-1 antigen would be to perform a large scale purification of 400ml conditioned medium. In this experiment the 10-1 antigen was eluted with 1% SDS, the eluate was then dialysed against 50 mM Tris, pH 9.0, and the detergent removed. The processed eluate was then dialysed against water, lyophilised, dissolved in non-reducing sample buffer, subjected to SDS PAGE electrophoresis on a 30cm gel

and blotted. The PVDF membrane was cut in two and one-half probed with the 3A7 Mab and the other used for N-terminal sequencing analysis. Western blotting revealed that as with cell lysate the elution was not totally effective, because quantities of the 10-1 antigen still remained attached to the protein G-DMP-3E12 beads (Fig. 30, compare 10-1 antigen migration at 55-60 kDa, lanes C and E). However, significant amounts of the 10-1 antigen had been purified using this procedure and was seen as a broad band migrating at approximately 55-60 kDa on both the western blot (Fig. 30, lane E) and commassie stained PVDF membrane (Fig. 30, lanes G,) which corresponds to a similar band recognised on cell lysate (Fig. 30, lane A). There was non-specific binding of proteins of 20-34 kDa and 40 kDa in size similar to that observed in Figure 29, and also of proteins 70-100 kDa in size which perhaps reflects the greater volume of conditioned media used. The remaining elution material in lanes G was prepared for N-terminal sequence analysis at the Biotechnology Resource Laboratory, Protein Sequencing and Peptide Synthesis Facility, Medical University of South Carolina. N-terminal sequencing revealed a six amino acid sequence R-P-K-H-P/V-I which via a Protein Database Search corresponded to the following proteins:

1. Human adenomatous polyposis coli protein (APC Protein)
2. Bovin alpha-S1 casein precursor
3. Alpha-S1 casein precursor (ALPHA-S1-CN)(Variants A, B, C, D, E and F)
4. Sheep alpha-S1 casein precursor.

Further purifications with different method variations and greater quantities of conditioned medium were performed, but a longer amino acid sequence was not obtained.

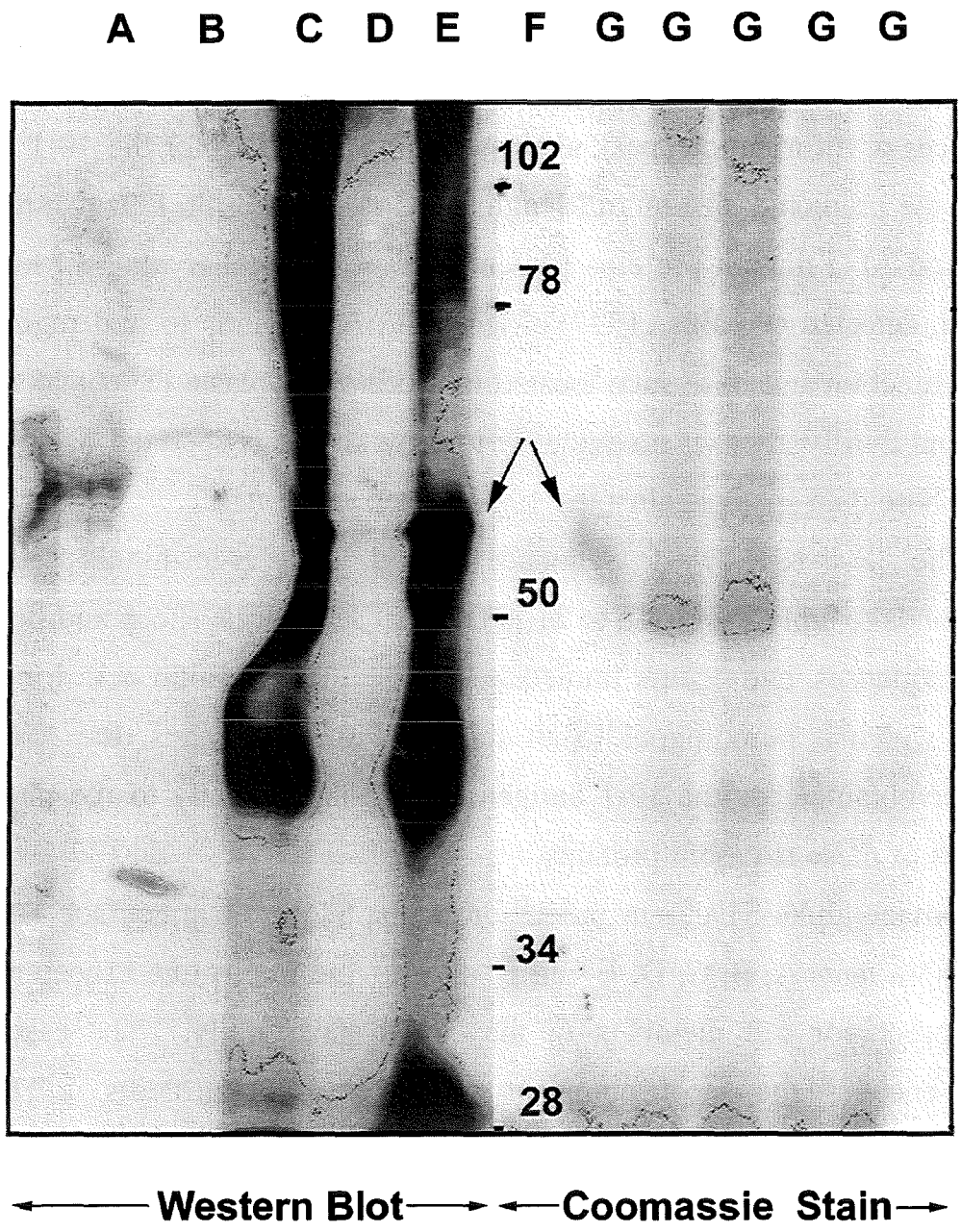


Figure 30. Purification of the 10-1 antigen. 10-1 antigen was eluted with 1% SDS, the eluate was then dialysed against 50 mM Tris, pH 9.0, the detergent removed, dialysed against water, lyophilised, dissolved in non-reducing sample buffer and subjected to SDS PAGE electrophoresis on a 30cm gel. The gel was blotted and one half probed with the 3A7 Mab and the other used for N-terminal sequencing. The strong band of 55-60 kDa was chosen for N-terminal sequencing. The lanes represent **A**, 10AS cell lysate; **B**, 10AS conditioned medium; **C**, protein G-DMP-3E12 beads after elution; **D**, protein G-DMP-3E12 beads only; **E**, aliquot of final elution material; **F**, molecular weight markers; and **G**, final elution material. The gel distortion in lane B is due to albumin present in the conditioned medium, and lanes F to G due to stretching of the gel during the western blot procedure. The arrows refer to purified 10-1 antigen visualised on the western blot and coomassie stained membrane.

Identification of the 10-1 antigen

Adenomatous polyposis coli protein (APC, Shoemaker et al., 1997) has been inarguably linked to the genetic progression of colon cancers. However, two pieces of information speak against it being candidate for the 10-1 antigen. Firstly, the predicted size of full-length APC is around 300 kDa (Smith et al., 1992) and APC mutant proteins present in cancers smaller than 80 kDa have not been detected (Polakis, 1997). Secondly, APC binds to and regulates β-catenin function and is as such located in a cell's cytoplasm (Shoemaker et al., 1997) and thirdly, there is no evidence to show that it is secreted or shed from cells. These data speak against the 10-1 antigen being APC.

The R-P-K-H-P/V-I sequence found by N-terminal sequencing corresponds to amino acids 16 to 21 in the goat sequences, sheep and bovine α-casein sequences, and occurs immediately after the 15-amino acid long signal peptide which is homologous to all species (Fig. 31). Thus, the N-terminal sequence obtained for the 10-1 antigen corresponds exactly to the mature N-terminus of these α-casein proteins.

Bovine alpha-S1 casein precursor, alpha-S1 casein precursor (ALPHA-S1-CN)(Variants A, B, C, D, E and F, also bovine family) and sheep alpha-S1 casein precursor are members of the casein superfamily. The casein gene family encodes the most abundant group of milk proteins. They are synthesised in the mammary gland as large precursors, split by proteases and secreted by both the constitutive and regulated pathways (Fiat and Jollès, 1989; Turner et al., 1992). In most species they are present in three forms alpha (α-casein), beta (β-casein), and kappa (κ-casein), and have the apparent molecular weights of 42 kDa, 25 kDa, and 22 kDa, respectively (Rosen et al., 1975). In the rat there is a fourth casein, gamma (γ-casein) which has a calculated molecular weight of 18 kDa (Nakhasi et al., 1984). Acknowledging the characteristics of the alpha-caseins and the properties of the 10-1 antigen it seemed that the identity of the 10-1 antigen could be best fitted with this protein group.

Only one mRNA species for rat α-casein has been described (Richards et al., 1981). This mRNA does not encode the R-P-K-H-P/V-I sequence after the signal peptide. However, two species of rat α-casein proteins α_1 and α_2-casein have been reported at the protein level through detection with monoclonal antibodies against rat α-casein (Blum et al., 1987; Kaetzel and Ray, 1984). As such, this sequence data particular to the α-casein proteins could suggest that in the rat there are multiple forms of α-casein. Therefore, the 10-1 antigen could be a new casein protein not yet characterised, or that this sequence belongs to a casein-like protein, or a completely new and novel protein.

peptide cleavage point

	1				50
Rat	MKLLILTCLV	AAALALPRAH	RRNAVSSQTQ	QEN..SSSEE	QEIVKQPKYL
Mouse	MKLLILTCLV	AAAFAMPRLH	SRNAVSSQTQ	QQH..SSSE.	.EIFKQPKYL
Goat	MKLLILTCLV	AVALA**RPKHP**	**I**NHQ.....	.G.....LS	PEVLNE.NLL
Goat	MKLLILTCLV	AVALA**RPKHP**	**I**NHQ.....	.G.....LS	PEVPNE.NLL
Sheep	MKLLILTCLV	AVALA**RPKHP**	**I**KHQ.....	.G.....LS	PEVLNE.NLL
Bovine	MKLLILTCLV	AVALA**RPKHP**	**I**KHQ.....	.G.....LP	QEVLNE.NLL
Pig	MKLLIFICLA	AVALARPKPP	LRHQEHLQNE	PD.....SR	EELFKERKFL
Guinea pig	MKLLILTCLV	ASAVAMPKFP	FRHTELFQTQ	RGGSSSSSSS	EERLKEENIF
Rabbit	MKLLILTCLV	ATALARHKFH	LGHLKLTQEQ	PE.....SSE	QEILKERKLL

Figure 31. The first 50 amino acid sequence of α-casein from rat, mouse, goat (2 sequences exist for goat), sheep, bovine, pig, guinea pig and rabbit species. The sequences were attained from the *Munich Information Centre for Protein Sequences.*

Examination of rat α-casein expression.

To deduce if rat α-casein is transcribed by BSp73AS pancreatic carcinoma cells a DNA probe of rat α-casein was made from mRNA purified from rat mammary glands and PCR performed with primers corresponding to the N- and C-terminal portions of rat α-casein (Fig. 32A). The cell lines were cultured in 2% FCS RPMI and 10% FCS RPMI, mRNA made, electrophoresed on a 1% agarose-formaldehyde gel and a Northern blot performed (Fig. 32B). The blots were probed under high and low stringency conditions. No binding of the rat α-casein probe was seen for any of the cell lines under either hybridisation condition, demonstrating that rat α-casein is not transcribed by

these cells. It is clear then that the R-P-K-H-P/V-I sequence is associated with another protein, a protein whose mRNA has low homology with the presently cloned rat α-casein mRNA. It could thus be a new member of the casein protein family or a novel protein.

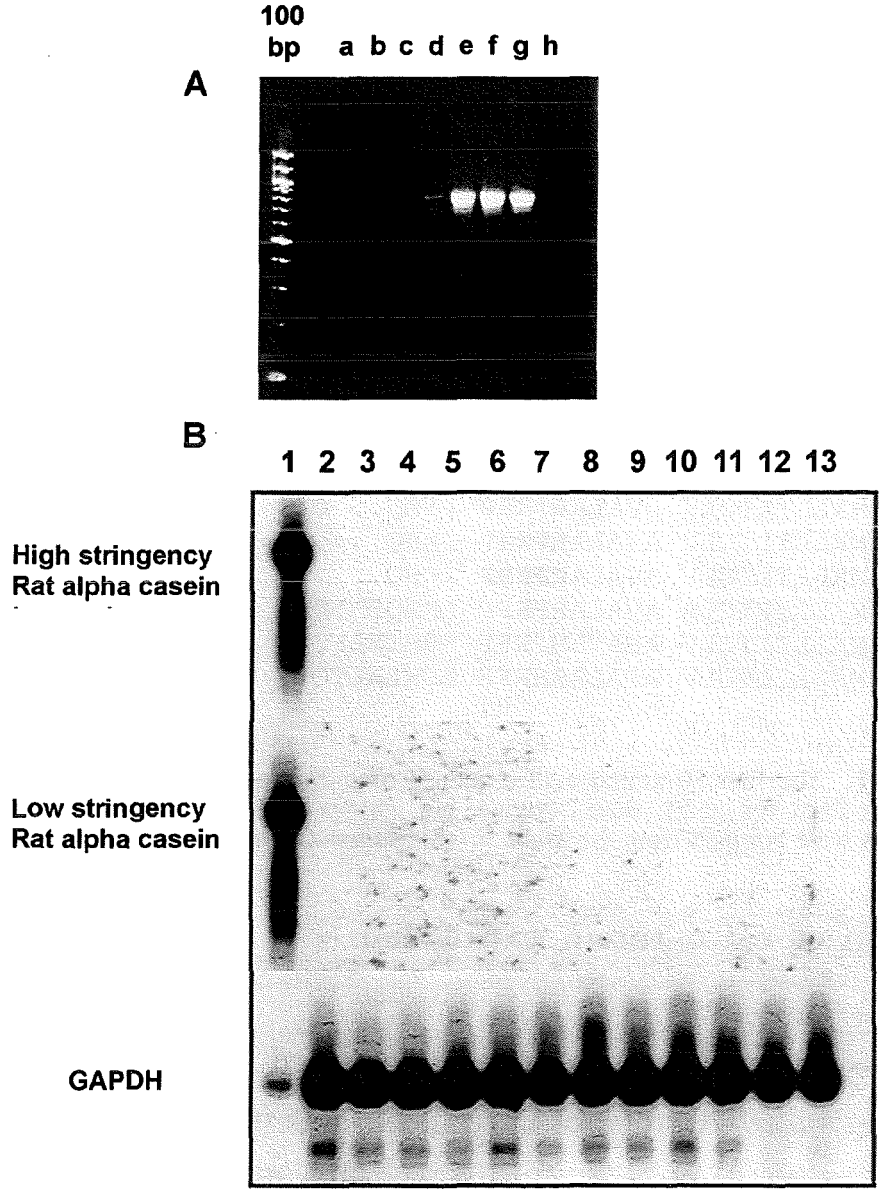


Figure 32A and B. A. Generation of rat α-casein probe. PCR was performed as described in Materials and Methods. The lanes correspond to the 100 base pair ladder (100bp), different magnesium concentrations of: a (0μM), b (125μM), c (250μM), d (375μM), e (500μM), f (625μM), g (750μM) and h (water control). **B.** Northerns of BSp73AS pancreatic carcinoma cell system. Northerns were probed with the rat-alpha casein probe under high and low stringency conditions, and with GAPDH. Clones from BSp73AS pancreatic carcinoma cell system were grown in 2%FCS RPMI (2%) or 10%FCS RPMI (10%). The lanes correspond to mRNA from: 1 (rat mamary glands), 2 (OAS, 2%), 3 (OAS, 10%), 4 (1AS, 2%), 5 (1AS, 10%), 6 (3AS, 2%), 7 (3AS, 10%), 8 (7AS, 2%), 9 (7AS, 10%), 10 (10AS, 2%), 11 (10AS, 10%), 12 (ASML, 2%) and 13 (ASML, 10%).

DISCUSSION

Reasoning that there are cell surface molecules expressed on tumour cells which are functionally associated with the generation of a metastatic phenotype, I set out to find and characterise such molecules and understand how they facilitate tumour cells to become metastatic. I have utilised subtractive immunisation to exploit the differences between non-metastasising and metastasising tumours to increase the chances of attaining monoclonal antibodies that specifically recognise metastasis-associated molecules. Two immunosubtractions have been performed with rat mammary tumours and rat pancreatic carcinoma cell lines of different metastatic potential. The first immunosubtraction with rat mammary tumour material was found to be optimal but not complete. The second subtractive immunisation used cell lines from BSp73 rat pancreatic tumours and it produced four antibodies, the 10-1 antibodies, that recognise a group of proteins termed the 10-1 antigen which are differentially expressed by the metastasising cell line. The 10-1 antigen is also expressed on other rat carcinoma cell lines of differing metastatic potential. It is actively secreted from the metastasising cell line in response to changes in growth conditions, and appears to be localised to filopodia, suggesting a role in tumour cell invasion. The secretion in part explains why the 10-1 antibodies did not inhibit tumour growth and metastasis in spontaneous metastasis assays. Purification and microsequencing of the 10-1 antigen revealed that it may be carried by a α-casein or casein-like protein. These data suggest that α-casein proteins apart from having roles in normal physiological processes may also play a part in abnormal pathological processes such as tumorigenesis.

The traditional approach to find new cell surface molecules involved with metastasis has been to inject metastatic tumour cell lines or tumour material into mice, and thereby generate monoclonal antibodies against cell surface antigens on tumour cells. The hope then is to screen the antibodies and

find one that recognises a molecule associated with metastasis. For example the metastatic 10AS cells could be simply used to immunise mice. However, the problem with such an approach is that during an immune response antibodies in the main are produced only against immunodominant epitopes (Golumbeski and Dimond, 1986). Thus in a pool of molecules where immunodominant and less-immunodominant molecules are present, antibodies are seldom produced against the less abundant or less-immunodominant molecules (Williams et al., 1992). The surface of tumour cells like 10AS cells will exhibit a plethora of immunodominant molecules and less-immunodominant molecules, and it is likely that sub-dominant epitopes are those important for contributing to the metastatic phenotype (Brooks et al., 1993). Therefore using the traditional approach with 10AS cells would greatly reduce the chances of attaining antibodies against rare and less-immunodominant molecules that are functionally involved with metastasis.

I therefore considered another method, subtractive immunisation, which focuses the immune reponse against epitopes which are different between two groups of antigens. This method also takes into consideration the variability in metastatic properties of cell subpopulations isolated from a tumour, which has important implications for the analysis of tumour cell surface molecules and their relationship to metastasis. In such analyses one must utilise cells that are highly metastatic and compare them with non-metastatic or poorly metastatic tumorigenic subpopulations isolated from the same primary tumour (Nicolson, 1984). This procedure is based on the ability of cyclophosphamide to kill proliferating B lymphocytes (Razzaque Ahmed and Hombal, 1984). It suppresses the immune system to immunodominant molecules and increases the likelihood of obtaining a humoral response against rare and less-immundominant molecules in a second round of immunisation. Previous authors have utilised subtractive immunisation to find cell surface molecules which are functionally associated with metastasis (Brooks et al., 1993; Sleeman et al., 1998). Similar stratigies have also been employed to find rare antigens in the frog egg (Denegre et al., 1997) and molecules involved with neurite

outgrowth (Matthew and Sandrock, 1987; Carnahan and Patterson, 1991). In subtractive immunisation the aim is to remove the immune response against antigens common to both the non-metastasising and metastasising tumours or tumour cell lines. This was performed by immunisations of non-metastatic variant cells (tolerogen; MTW9B for MTW9 tumours; 1AS for BSp73ASML cells) and the resulting humoral response was suppressed by cyclophophamide, after which related metastatic cells (immunogen; MT450 for MTW9 tumours; 10AS for BSp73ASML cells) were injected and an immune response was generated against epitopes present only on the immunogen.

In the initial subtraction that I performed using MT-W9B and MT450 tumour material, it was not clear whether such an immunosubtractive scheme could be sucessfully applied to tumour material, because of the complexity of cell types and tissue epitopes within the tumour. It was thus important to derive an estimate of the effectiveness of this immunosubtraction. My results indicate that the subtraction was effective, with the majority of the animals giving a differential humoral response to the metastasising MT450 tumour material. However, it was not complete, for initial hybridoma screening performed using tumour cells isolated from MTW9B and MT450 tumours found three of the eight Mabs were not specific for MT450 tumours. Later screenings (western blots, immunoprecipitations and immunofluorescence) with the MT450 tissue culture cell line and newly isolated MTW9B tissue culture cell line revealed further deficiencies in the specificity of these antibodies. For example, in immunofluorescence studies it was found that the antibodies had non-differential specificity.

The analyses performed with MTW9B tissue culture cell lines may have not been optimal, for after this data was obtained it was found that the MTW9B cell line metastasised, as such it did not reflect the phentoype of MTW9B tumours. Thus, using tissue culture cell lines as targets in these assays while having initially used tumour material as the tolerogen and immunogen for

immunisation may have given misleading information about the specificity of the subtractive antibodies. It may have been more appropriate to use tumour material in these experiments.

Nevertheless, histochemical studies confirmed earlier observations from cell ELISA using tumour cells isolated from tumours as targets, that the subtractive immunisation was not complete. The staining of the antibodies on paraffin sections from MTW9B and MT450 tumours was localised to the stromal regions of tumour sections from both tumours, demonstrating that they recognised immunodeterminants not associated with a metastatic phenotype. This observation in itself may be interesting for a variety of molecules that are connected with metastasis are localised to stromal regions of tumours, for example MMPs (Sako et al., 1994). However, after observing that the stromal staining with these antibodies was not specific to metastatic tumours in the MTW9 tumour family and other unrelated tumour models, further studies were not undertaken.

In contrast to the subtractive immunisation using tumour material, the subtractive immunisation using the 1AS and 10AS cells which proved to have stable metastasising properties was effective and complete, since none of the hybridomas recognised 1AS cells alone (tolerogen) and the four Mabs examined in detail were specific to the 10AS cells (immunogen). These data also indicate that a minimum of 10.3% (4/39) of the hybridomas generated through immunosubtraction are genuine products of subtraction. This is a high success rate which supports the use of this strategy to identify less-immunodominant molecules. Two different subclasses of antibodies were generated and they bound epitopes of the same antigen, the 10-1 antigen, which strongly suggests that the 10-1 antigen is greatly up-regulated on the 10AS cells in comparison to 1AS cells.

It must be said that 39 hybridomas from two mouse spleens is demonstrative of a low number of fusions between splenocytes and mouse myeloma cells. This can be explained by the use of a two-round injection procedure of cyclophosphamide (Sleeman et al., 1998), which kills more splenocytes than the once-only cyclophosphamide protocol (Brooks et al., 1993), thus reducing the number of viable B cells for fusion with the mouse myeloma cells. However, studies using the once only cyclophosphamide injection gave rise to a greater number of hybridomas, but a smaller percentage are genuine products of the subtraction (Denegre et al., 1997). In order to suppress the anticipated larger number of immunodominant epitopes present in tumour material and to increase the chances of attaining more antibodies specific to the immunogen, a two-step cyclophosphamide injection procedure was used in this thesis. In effect, my results suggest that in subtractive immunisation the specificity of the immune response against epitopes expressed specifically on the immunogen is inversely proportional to the number of hybridomas generated. Cyclophosphamide treatment can be varied to affect this relationship. Recent studies using the two-round injection procedure have achieved success (Sleeman et al., 1998).

The 10-1 antigen presents in different molecular weight forms. They could be the result of differential post-translation modifications that occur in the Golgi by the addition of N- and/or O-linked glycosylation, or alternative splicing of pre-messenger RNA, or be the products of some form of proteolytic processing. Studies were performed to test if the 10-1 antigen was modified by glycosylation, none were found. As the 10-1 antigen could be an α-casein or casein-like protein, it would be interesting to consider if differential splicing is a characteristic of these proteins. Indeed, it has been observed that the synthesis of multiple protein variants exists for the α-caseins in bovine, caprine and ovine milks (Bouniol et al., 1993; Ferranti et al., 1997; Boisnard et al., 1991). This is not only restricted to the α-caseins, exon skipping has also been recorded for human β-caseins (Menon et al., 1992).

When further studies firmly link the identity of the 10-1 antigen to that of an α-casein or casein-like protein, investigations may be performed to consider if differential splicing is responsible for the many forms of the 10-1 antigen.

Another possible explanation for the multiple forms of the 10-1 antigen is proteolytic processing, for in confocal microscopy experiments it was localised to vesicle-like structures. Many proteins secreted by the regulated branch of the secretory system are proteolytically processed and activated in secretory granules. Processing can involve the removal of an N-terminal propeptide (Fritz et al., 1986; Pratt et al., 1986) or short spacer peptides from polyprotein precursors (Comb et al., 1982). In the latter case cells in different tissues can process the precursors to give different "mature" forms. An example of such a protein is prosomatostatin, which is processed to a 28 amino-acid form by cells in the gut and a 14 amino-acid from by the brain and pancreatic cells (Noe et al., 1986). In fish pancreatic islet tissue there are two proteases which process prosomatostatin to give products of different lengths (Mackin and Noe, 1987). The many forms of the 10-1 antigen could be an example of such a proteolytic process.

The 10-1 antibodies recognise epitopes showing strong localisation to filopodia and secretory vesicle-like structures within cells. Investigations found that the 10-1 antigen was secreted from 10AS cells, and that growth conditions affected secretion levels, demonstrating perhaps that a regulated secretion system is in operation, and that the secretory vesicle-like structures may carry the 10-1 antigen to the surface of 10AS cells for release. This is an important finding for it is a long standing belief that the release of cell surface components or molecules *in vivo* may be important in assisting the survival of neoplastic cells. Indeed, many highly metastatic cells often shed/secrete more tumour cell antigens than that from their low metastatic counterparts (Alexander, 1974; Kim et al., 1975). Antigens shed by malignant cells are normal, but often incomplete, blood group substances, foetal antigens, and

tissue specific antigens (Nicolson, 1982, 1988; Black, 1980). It must also be said that the release of material from a cell can also just be a normal cellular process that is not strictly associated with malignancy (Nicolson, 1984).

A similar feature of the 10-1 antigen and alpha casein proteins is that they are secreted. In mammary epithelial cells caseins are believed to be secreted by both the consititutive and regulated pathways (Turner et al., 1992). Previous work on casein protein secretion found that nocodazole, a anti-microtubule drug could partially inhibit their secretion (Rennison et al., 1992). Nocodazole had no effect on the secretion of the 10-1 antigen (data not shown), suggesting that the 10-1 antigen is secreted by a non-conventional secretory process. There are a number of proteins secreted by such mechanisms, for example RAS-like GTP binding proteins such as SEC4 (Salminen and Novick, 1987) and RAS2 (Deschenes and Broach, 1987), FGF-1 (Shi et al., 1997) and Interleukin-1 (Hazuda et al., 1991). Therefore, considering the differences in the secretory mechanisms of the 10-1 antigen and casein proteins, this may suggest that the N-terminal sequence obtained for the 10-1 antigen belongs to a genera of proteins other than caseins. However, it has been speculated that casein vesicles cannot necessarily be regarded as being similar to regulated secretory vesicles (Turner et al., 1992), for the effects of nocodazole are not complete. Thus, caseins could also be released by another unique secretory pathway. Another point of contention is that the effects of nocodazole on protein secretion have not been seen in all cell types (Schroer and Sheetz, 1991) and as such these observations would require further investigation with other secretory inhibitors.

The identification and characterisation of the proteins bearing the 10-1 antigen in 10AS cells is critical to understand any role they might play in tumorigenesis and metastasis. Because of the multiple forms of the 10-1 antigen and having no understanding of how they are related, and that some of these forms are expressed on 1AS cells, an affinity purification procedure was

chosen ahead of the construction and screening of a cDNA library. Purification of the 10-1 antigen was made difficult by the very high affinity of the 10-1 antibodies for the 10-1 antigen. I therefore had to use harsh elution conditions to elute the 10-1 antigen, which resulted in considerable leaching of antibodies and the release of other non-specifically bound proteins from the protein G-DMP-Mab colums. Despite the ensuring difficulties experienced with the affinity purifcation, a limited N-terminal sequence was obtained for one of the 10-1 antigen proteins (R-P-K-H-P/V-I) that corresponds to the N-terminal residues of certain α-casein proteins. This sequence occurs immediately after a 15 amino-acid long signal peptide which is homologous to α-casein proteins found in all species, suggesting that the 10-1 antigen is a new α-casein protein or a casein-like protein.

The complete sequence of rat α-casein has been determined and encodes a 15-amino acid long signal peptide and mature protein of 269 residues (Hobbs and Rosen, 1982). However, the N-terminal sequence for the 10-1 antigen protein is not contained in this sequence. Nevertheless, multiple subtypes of α-casein have been identified in other species. These have not yet been cloned in the rat. Investigations to ascertain whether the mRNA for the cloned rat α-casein is expressed in 10AS cells clearly showed that none is present. However in many casein species only three regions of the casein mRNAs are conserved: i) the 5′ non-coding region; ii) the signal peptide coding region; and iii) the region encoding the phosphorylation sites. These regions correspond to the functional role of caseins, namely: i) to be secreted; ii) to form proteins aggregates termed micelles; and iii) to be phosphorylated to allow Ca^{2+} binding and transport. Analysis of rat, bovine and guinea pig cDNA sequences have demonstrated considerable divergence among the individual members of the casein gene family (Jones et al., 1985). They have in fact been grouped among some the most divergent of protein families studied. Thus if the protein bearing the 10-1 antigen was a new rat α-casein or casein-like protein present in 10AS cells and had undergone a series of deletion/insertion or splicing

events, then a DNA probe for rat α-casein which has only homology with these three conserved regions may not hybridise to the mRNA encoding the protein. An approach to find if other casein or casein-like proteins exist in 10AS cells would be to design primers for these three homology areas and perform PCR to see any DNA products can be attained. If this is the case then one could go about cloning these new casein protein species. Such a study is currently underway.

There is the possibility that the 10-1 antigen is not carried by a casein or casein-like protein. No other proteins apart from APC and α-caseins were found to contain the same N-terminal sequence. An important observation in initial studies with the 10-1 antibodies was that the 10-1 antigen is localised to filopodia of 10AS cells. There are a number of known molecules that are localised in this manner and are associated with tumour cell invasion, such as urokinase plasminogen activator and cathepsin D. However, they do not have similar biochemical properties as the proteins recognised by the 10-1 antibodies, and do not contain the N-terminal sequence obtained for the 10-1 antigen. The proteins of best fit are the caseins. Thus, future work will concentrate on genetic approaches, for example the screening of a bacterial expression library with the 10-1 antibodies to isolate cDNAs encoding the 10-1 antigen protein. This will confirm whether or not it is a casein or casein-like protein that expresses the 10-1 antigen.

In studies performed here there is up-regulation of an antigen, the 10-1 antigen, containing a N-terminal epitope that probably belongs to α-casein proteins, on neoplastic cells originating from the rat pancreas. *In vivo* functional studies were performed with the 10-1 antibodies to see if they could inhibit the action of protein(s) bearing 10-1 antigen epitopes. No effect by the 10-1 antibodies on tumour growth or metastasis was observed. This result is contrary to previous immunosubtractions which have generated antibodies that suppress metastasis (Brooks et al., 1993; Sleeman et al., 1998). However, this

is likely to be a consequence of the 10-1 antigen being secreted, or because the 10-1 antibodies bind to a non-functional domain of proteins harbouring the 10-1 antigen.

Studies to consider the expression of the 10-1 antigen in 10AS tumours and their metastases found that it was down regulated and expressed in a lower molecular weight form than that seen in the 10AS cell line. The most likely reason for this altered expression is that cells in tumours experience different growth conditions as compared to those grown in tissue culture. In tissue culture there is a continuous source of oxygen, and a plentiful supply of growth factors and nutrients provided by FCS. In a tumour, the growth conditions are not so nutritious, for example the environment is hypoxic and there is a different supply of stimulatory factors. Cells in tumours are affected by these differences and alter their expression of proteins and receptors, accordingly. The down regulation of the 10-1 antigen *in vivo* could be an example of such processes.

Is the *in vivo* 10-1 antigen expression related to metastasis? It was found that the 10-1 antibodies had no effect on tumorigenesis, so one could simply say the 10-1 antigen is not associated with metastasis. However, investigations to consider if the 10-1 antigen is correlated with metastasis found that it was expressed by the majority of rat mammary and prostate carcinoma cell lines considered. Admittedly, there was not a strict correlation between its expression and a metastatic phenotype, however these observations still suggest that the 10-1 antigen may be connected with tumour growth and metastasis but in a non-obligatory way. Thus, from this work no direct functional association of the 10-1 antigen with metastasis can be made, however, it cannot be ruled out. It is only through further ongoing work to deduce the identity of protein(s) bearing the 10-1 antigen epitope(s) can conclusive information about its involvement in metastasis be found.

Previous studies have provided evidence of casein or casein-like protein expression in several tissues and neoplasms. Expression of these proteins have been observed in the pancreas and other tissues rather than just the mammary gland (Onoda and Inano, 1997; Barash et al., 1995; Pich et al., 1976). Studies have noted the up-regulation of casein or casein-like material in mammary carcinoma (Herbert et al., 1978; Bussolati et al., 1975; Medina et al., 1987; Smith et al., 1984; Cohen et al., 1987; Rudland et al., 1993), extramammary Paget's disease and carcinoma of the lung, the endometrium and the gastrointestinal tract (Cohen et al., 1993; Suzuki et al., 1998).

Acknowledeging that there is a large body of evidence showing that casein or casein-like proteins are present in neoplasms, one must ask how could they be functionally involved in driving or facilitating carcinogenesis? There are observations from their effects on lymphocytes that could link casein or casein-like proteins with carcinogenesis. They have been shown to be responsible for inducing neutrophil chemotaxis (Wilkinson, 1972; Van Epps, 1977; Wilkinson, 1988), neutrophil migration into the peritoneal cavity (Balcom, 1985), affecting lymphocyte proliferation (Coste, 1992), and influencing phagocytic function (Russell, 1975). A mode by which casein or casein-like proteins could cause these responses in lymphocytes is through interactions with integrins. Casein proteins have been shown to be bound by $\alpha_M\beta_2$ (Mac-1), $\alpha_X\beta_2$ (p150,95) and $\alpha_4\beta_1$ integrins (Davis, 1992; Davis et al., 1997). The expression of $\alpha_M\beta_2$ and $\alpha_X\beta_2$ integrins is restricted to monocytes, macrophages, and granulocytes (Relman et al., 1990), and they are imporant for leukocyte transmigration from blood vessels (Springer, 1990). $\alpha_4\beta_1$ integrin is expressed on lymphocytes and has been shown to be involved in mediating their adhesion to the ECM and to other cells such as endothelial cells and leukocytes (Lobb and Hemler, 1994; Kilger and Holzmann, 1995; Hynes, 1992; Hemler et al., 1987; Guan and Hynes, 1990; Komoriya et al., 1991; Pulido et al., 1991).

Using these cell adhesion molecules leukocytes are able to invade organs and tissues in response to chemotactic signals that occur as a result of tissue

injury or infection. It is believed that lymphocytes are recruited to tumours by similar exogenous signals or tumour-secreted cytokines (Melani et al., 1995). In the tumour lymphocytes can either recognise and kill neoplastic cells and/or elicit positive influences on tumour growth and vascularisation (Mantovani et al., 1991). These actions are regulated by the balance of inhibitory and stimulatory signals produced by both malignant and infiltrating cells. Thus, seeing that casein or casein-like proteins cause chemotaxis in leukocytes and that they are bound by $\alpha_M\beta_2$, $\alpha_X\beta_2$ and $\alpha_4\beta_1$ integrins, they could play a role in carcinogenesis by recruiting leukocytes to tumours. These leukocytes may then through the net balance of tumour-host interactions influence tumorigenesis.

In conclusion, I have successfully used subtractive immunisation to find antigens specifically expressed on metastasising cells. Four antibodies were found that recognised the same group of proteins, encoding the 10-1 antigen, and these antigens are greatly up-regulated on the metastasising 10AS cell line used in the immunisation. Characterisation of the 10-1 antigen revealed that it has interesting biochemical properties which are consistent with a role for it in neoplasia. Interestingly, the N-terminal microsequence of a purified protein bearing the 10-1 antigen suggested that proteins encoding the 10-1 antigen may be related to the casein protein family. As caseins interact with integrins expressed on leukocytes whose presence in tumours may effect tumour cell proliferation and metastasis, it is fascinating to speculate that the expression of the proteins containing the 10-1 antigen may positively influence tumour growth. Studies are currently underway to completely deduce the identity of proteins bearing the 10-1 antigen to confirm their nature and function.

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RESUME

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Publications

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Sleeman, J.P., **Hebbard, L. W.**, Zawadzki, V., Hofman, M., Ponta, H. and Herrlich, P. 1998. (In preparation). Hormonal regulation of CD44 expression in the mammary gland.